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Award Number: DAMD17-00-1-0442

TITLE: Development of a Novel Test System for Screening for Antagonists of ErbB Receptors in Breast Cancer

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Development of a Novel Test System for Screening for Antagonists of ErbB Receptors in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0442

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The focus of this project is to develop a system for screening for novel drugs and drug targets to inhibit the progression of breast cancer. Our approach capitalizes on recent findings in our laboratory that should allow new types of screens for drugs. These screens should enable the identification of drugs that arrest the growth of breast cancer cells as well as the identification of new drug targets. Specifically, certain growth factor receptors will be targeted that are known to be present in abundance on the surface of breast cancer cells. These receptors have been designated as the ErbB family of receptors. Cancers expressing an abundance of one of these receptors, the ErbB2 receptor, have been shown to be resistant to conventional therapy. Very recently a drug that targets ErbB2 directly has been found to increase patient survival in a phase III clinical trial. The identification of additional drugs or small molecules that target this family of receptors and impair their function should greatly enhance our ability to successfully treat patients with this form of cancer.

14. SUBJECT TERMS
breast cancer, therapy, novel drug screen, ErbB receptor dimerization antagonists

15. NUMBER OF PAGES
39

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Multiple common carcinomas frequently express high levels of the EGF family of tyrosine kinase growth factor receptors (EGFR/ErbB1, ErbB2, ErbB3). Specifically, ErbB-2 is upregulated in a high percentage of breast cancer tumors. The frequency of Erb-B2 overexpression indicates this may be an early and often necessary change for successful tumor engraftment. Erb-B2 is a receptor tyrosine kinase which activates multiple intracellular pathways mainly associated with cell proliferation and survival. Recent evidence shows therapies that block Erb-B2 signaling are effective anti-cancer agents. The EGFR, and Erb-B3 are also upregulated, although to a lesser degree, in mammary carcinomas. The mechanism by which Erb-B2 receptor mediates its effects is complicated by the ability of the EGF family of receptors to heterodimerize and activate different signaling pathways. The general aims of this research are to develop a high-throughput screen for antagonists of signaling events mediated by the EGF family of receptors, and to identify novel proteins that associate with the receptors as potential drug targets. To accomplish these goals we are applying a technique pioneered in our laboratory based on enzyme complementation. This approach involves the construction of chimeric proteins consisting of two proteins of interest which dimerize, for example Erb-B2 and the EGFR, and two inactive portions of an enzyme. The dimerization of the proteins forces the complementation of the enzyme resulting in an increase in enzyme activity which can be assayed by fluorescence or light emission and can be performed in 96-well format.

Body

Assays for homodimerization of ErbB2 and heterodimerization with other EGFR family members

Initially we focused on monitoring the dimerization of the EGF receptor family members using β-galactosidase complementation. We constructed β-gal fusions to three of the four EGFR family members (EGFR, ErbB2, and ErbB3). These constructs were expressed pairwise in C2C12 myoblasts and their expression was confirmed by western blot.

Analysis of the EGFR (Blakely et al., 2000) demonstrated that homodimerization of the EGFR could be detected in as little as one minute after exposure of cells expressing the chimeric EGFR-β-gal fusion proteins to EGF. The dose response of these cells to different EGF-related ligands was consistent with the published affinities of these ligands for the EGFR. In addition, the addition of antibodies to the medium which were reported to block the ability of the EGFR to bind EGF, were able to effectively block the signal generated in the assay in the presence of EGF, further validating the application of this system to high throughput screening methods.

Although we have successfully monitored the homodimerization of another family member, ErbB2, the induction of b-galactosidase activity we were able to generate upon addition of ligand (~ 1.5-2-fold) was not sufficient for high-throughput screening methods. In addition heterodimers of ErbB2 and EGFR were undetectable in our assay. Therefore we have adopted a similar but different approach based on the signaling proteins which bind to the receptors when they become activated.

The EGF family of tyrosine kinases becomes phosphorylated on multiple tyrosine residues upon activation. These phosphorylations serve as docking sites for cytoplasmic, SH2 domain

containing adapter proteins such as Grb2. As an alternative to ErbB2 receptor dimerization we are pursuing the interaction of ErbB2 with Grb2 and Grb7, both of which are SH2 domain proteins which have been reported to interact with this receptor. Monitoring the interaction of one of these adapter proteins with the receptor is actually superior to monitoring its dimerization because it is indicative of the activation of that particular receptor. Antagonists identified in this manner may inhibit ligand binding, receptor dimerization, kinase activity, or association of the adapter protein with the receptor.

In order to determine whether it would be possible to monitor the interaction of a membrane-bound and cytoplasmic protein we utilized the FKBP12-FRB system. FKBP12 binds FRB only in the presence of the drug rapamycin. A three part fusion protein was constructed which included the extracellular and transmembrane domains of the EGF receptor fused to FKBP12 and the Δω β-galactosidase fragment (which represents the receptor being studied). As a model cytoplasmic interacting protein, FRB was fused to the Δα β-galactosidase fragment. C2C12 myoblasts were engineered to express both chimeric proteins and β-galactosidase activity was monitored in the presence and absence of rapamycin. We tested the membrane-bound/cytoplasmic system in several orientations, and in all but one orientation tested the induction of FKBP12-FRB dimerization resulted in a 5-15 fold induction of β-galactosidase activity. This result indicates it is possible to monitor protein interactions in this configuration. Further, an amino-terminal fusion to the Δα fragment and a carboxy terminal fusion to the Δω fragment exhibits a greater signal to noise ratio than the other orientations providing an optimal framework for experimental design purposes.

The EGF dependent interaction of Grb2 with the EGFR has been monitored through β-galactosidase complementation (Palmer, Michelle. Applied Biosystems, personal communication), indicating the feasibility of this approach and its application to ErbB2 activation.

Currently we have cloned all of the necessary EGF family receptors and Grb2 into retroviruses as fusions to the β-galactosidase fragments. The cDNA construct for Grb7 has been obtained and the associated β-galactosidase vectors are being made.

<u>Construction of a Mammalian Two-Hybrid System in Order to Identify Novel Targets for Breast Cancer</u>

In order to identify novel drug targets associated with the EGF family of receptors we proposed the development of a novel mammalian two-hybrid assay based on β-galactosidase complementation. The envisioned screen would consist of a cell line expressing ErbB2 fused to the Δα β-galactosidase fragment. A cDNA library will be constructed as fusions to the Δω fragment. The ErbB2 cell line will be transduced with the library. Only cells which have received a cDNA encoding a protein which interacts with the receptor will exhibit increased β-galactosidase activity. The positive cells will be rescued by flow cytometry through fluorescent staining for β-galactosidase activity.

To assay the sensitivity of the β-galactosidase system we attempted a "spiked" library screen. In this test system we used the tripartite fusion described earlier (EGFR extracellular and TM regions – FRB-Δα) to represent the receptor being studied. A cell line was created using this construct and an empty Δω vector was used as a mock library. Into the mock library, different concentrations of an interacting protein (FKBP12-Δω) were added in ratios ranging from 1:10-

1:10,000. The tripartite fusion cell line was then infected with the spiked library. Rapamycin was then added to the cells which induces the interaction of the FKBP12 and FRB domains. The transduced population was stained for β-galactosidase activity and the positive cells were isolated by flow cytometry. Only the ratios 1:10 and 1:100 yielded positive cells after four rounds of selection. We believe the apparent lack of sensitivity of the system is due to inefficient fluorescent β-galactosidase staining. The substrate must be loaded by hypotonic shock, and once cleaved rapidly leaks out of the cell and can enter neighboring cells. Numerous substrates were attempted with similar results.

To overcome this limitation, we created a similar complementation system based on the well-characterized TEM1 β-lactamase (Wehrman et al., 2002). The TEM1 β-lactamase gene was rationally divided based on non-contiguous structural elements. Several pairs were isolated which achieved interaction-dependent activity in bacteria. A specific pair (α197 and ω198) were chosen for further study. In order to improve the complementation observed for the β-lactamase fragments a peptide screen was undertaken to select peptides that would increase the complemented activity of the β-lactamase enzyme. The improved pair was then tested for its ability to monitor the interaction of FKBP12 and FRB in mammalian cells.

Several characteristics of the β-lactamase enzyme make it ideally suited for this type of complementation system. The protein is small (36 kDa), making each fragment less than 20 kDa which is smaller than green fluorescent protein (GFP). The enzyme is active as a monomer precluding the need to form higher order structures, and a robust cell-permeable substrate has recently been engineered. In mammalian cells the system performed extremely well in the fluorescence assay giving 50-100 fold increases in fluorescence upon dimerization of the fragments. In addition the fragments appear stable in mammalian cells, and we have shown it is possible to monitor interactions when one of the proteins is bound to the plasma membrane and the other cytoplasmic. Currently we are performing the previously described spiked library screen with the β-lactamase fragments to determine the applicability of this system to a library screen.

Key Research Accomplishments

- **Optimization of the β-galactosidase complementation system:** We have optimized the orientation of the fusion proteins used in the β-galactosidase system. We determined that fusion to the N-terminus of the Δα fragment, and C-terminal fusion to the Δω fragment consistently generate the highest signal to noise ratio, as well as the highest overall signal.
- **Interaction of a membrane-bound and cytoplasmic protein:** Using the FKBP12-FRB system we proved it is possible to monitor the interaction of a membrane bound and cytoplasmic protein.

- **Development of an alternative complementation system:** We engineered an alternative complementation system to monitor protein-protein interactions in live mammalian cells based on the TEM1 β-lactamase gene. The advantages of this assay include:
    1. 50-100 fold increase in signal to noise ratio based on the fluorescence assay
    2. small fragment size limits steric concerns
    3. rapid signal generation upon induction of dimerization

4. ability to monitor cytoplasmic and membrane-bound proteins

Reportable Outcomes

List of reportable outcomes should include manuscripts, abstracts, presentations, patents and licenses applied for an/or issued, development of cell lines, data bases, animal models…

Manuscripts: (copies included in Appendix)

Blakely, B.T., Rossi, F.M.V., Wehrman, T.S., Charlton, C.A. and Blau, H.M. (2002) Protein interactions in live cells monitored by β-galactosidase complementation. In Protein-Protein Interactions: A Molecular Cloning Manual, (E. Golemis, ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 407-414.

Wehrman, T.S., Kleaveland, B.M., Her, J-H., Balint, R. and Blau, H.M. (2002) Protein-protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments, Proc. Natl. Acad. Sci. USA 99:3469-3474.

Patent:

1997: Detection of molecular interactions by reporter subunit complementation, U.S. Patent No. 6,342,345 B1 - International Application No. PCT/US98/06648 – Stanford Docket S96-125 (issued 1/29/02).

Conclusions

Several experiments were performed to optimize the orientation specificity of the β-galactosidase fragments. We have successfully identified the most favorable orientations to fuse proteins to the β-galactosidase fragments, which in addition to making the system more straightforward, cuts the amount of work involved in studying an interaction by 3/4, and increases the probability of obtaining productive interactions.

Our efforts to monitor the dimerization of ErbB2, and its heterodimerization with other EGF receptor family members met with limited success. Therefore we have adopted an alternative strategy based on the interaction of ErbB2 and Grb2/Grb7. This approach is superior in that the actual activation of the receptor is indicated by the interaction, not solely dimerization. Drug screens using this system should reveal chemicals which can inhibit not only receptor dimerization but receptor kinase activity, and the interaction of downstream signaling components which are necessary for the mitogenic effects of the growth factor. Using the FKBP12-FRB system we have shown it is possible to monitor, via β-galactosidase complementation, the interaction of a membrane bound and cytoplasmic protein. Further validating this approach, the system was used successfully to monitor the interaction of the EGFR and Grb2.

Furthering the concept of protein fragment complementation systems, we have successfully adapted the β-lactamase gene to this technology. This system has several advantages to the β-galactosidase system such as: small fragment size; reducing concerns about steric hindrance of the interactions being studied, the enzyme is active as a monomer simplifying the interaction (as opposed to the tetrameric nature of β-galactosidase), and the availability of a recently developed cell-permeable fluorescent substrate makes it possible to generate 50-100 fold increases in

fluorescence upon protein dimerization. This technology enables the construction of a mammalian two-hybrid screen which would permit the identification of proteins that interact with receptors, which is currently not possible with existing systems.

References

Blakely, B.T., Rossi, F. M.V., Tillotson, B., Palmer, M., Estellés, A. and Blau, H.M. (2000) Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech. 18:218-222.

Wehrman, T.S., Kleaveland, B.M., Her, J-H., Balint, R. and Blau, H.M. (2002) Protein-protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments, Proc. Natl. Acad. Sci. USA 99:3469-3474.

Appendices (reprints)

Blakely, B.T., Rossi, F. M.V., Tillotson, B., Palmer, M., Estellés, A. and Blau, H.M. (2000) Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech. 18:218-222.

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Wehrman, T.S., Kleaveland, B.M., Her, J-H., Balint, R. and Blau, H.M. (2002) Protein-protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments, Proc. Natl. Acad. Sci. USA 99:3469-3474.

Epidermal growth factor receptor dimerization monitored in live cells

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Received 13 September 1999; accepted 30 November 1999

We present a method for monitoring receptor dimerization at the membrane of live cells. Chimeric proteins containing the epidermal growth factor (EGF) receptor extracellular and transmembrane domains fused to weakly complementing β-galactosidase (β-gal) deletion mutants were expressed in cells in culture. Treatment of the cells with EGF-like compounds for as little as 15 s resulted in chimeric receptor dimerization detectable as β-gal enzymatic activity. The dose response of chimeric receptors was ligand specific. β-galactosidase complementation was reversible upon removal of ligand and could be reinduced. Antibodies that block ligand binding inhibited receptor dimerization and β-gal complementation. These results demonstrate that β-gal complementation provides a rapid, simple, and sensitive assay for protein interactions and for detecting and monitoring the kinetics of receptor dimerization.

Keywords: Protein interactions, receptor dimerization, EGF receptor

Dimerization, or higher order oligomerization, of cell surface receptors is often a prerequisite for their activation and signal transduction. For example, EGF receptor homodimerization, as well as its heterodimerization with the related erbB2 receptor, are stabilized by the binding of several EGF-related peptide growth factors. Following ligand binding and dimerization, the receptor undergoes a conformational change that leads to its autophosphorylation and activation of the receptor tyrosine kinase[1–3].

Chemical crosslinking, immunoprecipitation, and fluorescence resonance energy transfer (FRET) have been used extensively to study receptor dimerization. However, biochemical methods require cell disruption and do not preserve all interactions. The yeast two-hybrid system[4] cannot be used for integral membrane proteins. More recently, methods such as FRET analysis of proteins tagged with green fluorescent protein (GFP) and complementation of β-gal and dihydrofolate reductase (DHFR) have made possible the detection of protein interactions in live mammalian cells[5–7].

We have shown previously that complementation of bacterial β-gal, first demonstrated in prokaryotes[8], can be used to detect cytoplasmic protein interactions in mammalian cells[6,9]. When two different, weakly complementing deletion mutants of β-gal, Δα and Δω, are fused to two interacting proteins and expressed in a cell, the interaction of the non-β-gal portions of the chimeric proteins drives β-gal complementation, and the resulting β-gal activity serves as a measure of that interaction. There are several advantageous properties of β-gal complementation: (1) it works in live mammalian cells; (2) it monitors interactions in the cellular compartment in which they normally occur; (3) the signal is amplified by the enzymatic activity of β-gal; (4) the signal can be quantitated by biochemical, histochemical, fluorescent, and chemiluminescent assays; (5) overexpression of complementing protein chimeras can be avoided, as low levels are readily detected[10,11].

Here we report that by using β-gal complementation to monitor membrane protein interactions, dimerization of the EGF receptor can be readily detected using rapid assays amenable to high-throughput screening methods. This system facilitates the study of signal transduction and should be useful for screening for agonists and antagonists of several receptors.

Results

Expression of EGF receptor–β-gal chimeric proteins. The weakly complementing Δα and Δω deletion mutants of β-gal were fused to the extracellular and transmembrane regions of the human EGF receptor to form chimeric receptor molecules (Fig. 1A). Deletion of the cytoplasmic domain of the receptor removes the sequences that lead to a decrease in receptor activity by either internalization or phosphorylation on serine and threonine residues[12]. The use of such a truncated receptor avoided internalization of the chimeric receptors, and the consequent decrease in surface expression, eliminating this variable from analyses of receptor dimerization over time. The two chimeric cDNAs were each cloned into retroviral vectors encoding selectable markers (Fig. 1B) and transduced into C2C12 mouse myoblasts. After selection with G418 and hygromycin, cells were treated with EGF, and β-gal enzyme activity was assayed in live cells using a fluorogenic substrate and the fluorescence-activated cell sorter (FACS). In cells expressing only one of the chimeric molecules, no change in β-gal activity was observed upon addition of EGF, demonstrating that β-gal mutants of one type cannot form an active β-gal enzyme (Fig. 1C, 1D).

In cells expressing both chimeric molecules, EGFR–Δα–β-gal and EGFR–Δω–β-gal, FACS analysis showed that β-gal activity was significantly induced by EGF. In the absence of EGF, the population of transduced cells consisted of a mixture of cells with low and high levels of β-gal activity (Fig. 1E, black curve), which was not unexpected as it is known that the EGF receptor can dimerize in the absence of EGF in cell lines, such as A431, that express high levels of the receptor[13]. Much of the subpopulation that had low β-gal activity underwent a ligand-dependent increase in β-gal activity following stimulation with EGF (Fig. 1E, red curve).

Complementation of β-gal is dependent on the specific interaction of the non–β-gal portions of the chimeric receptor, as coexpression of EGFR–Δω–β-gal with a chimeric protein that contained the

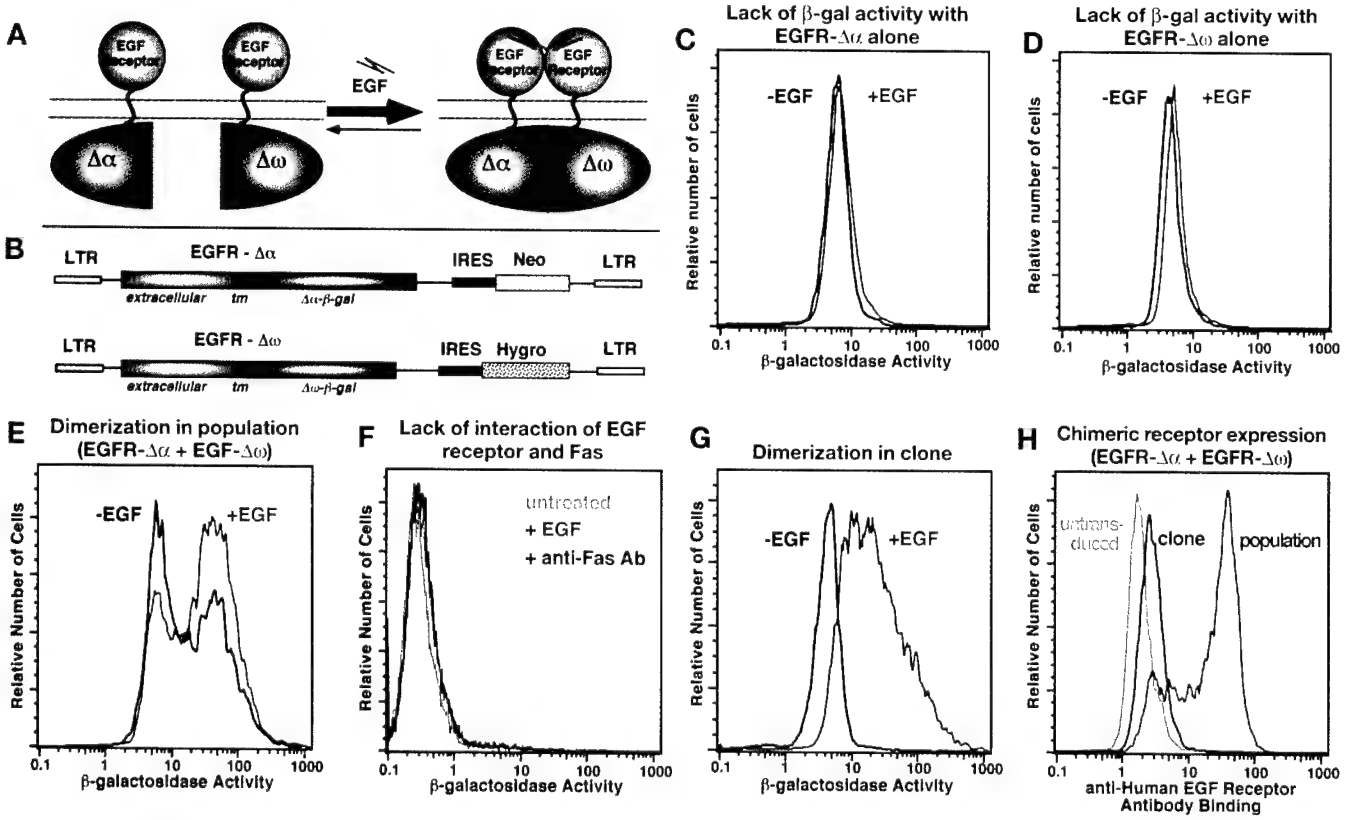

Figure 1. EGF receptor dimerization monitored using β-gal complementation. (A) Two weakly complementing deletion mutants of β-gal linked to the extracellular and transmembrane domains of the EGF receptor to determine whether receptor dimerization, which is stabilized by the addition of EGF, can drive β-gal complementation. (B) The human EGF receptor (EGFR) extracellular and transmembrane domains cloned 5′ to and in frame with the E. coli lacZ deletion mutants Δα and Δω in retroviral vectors expressing neomycin or hygromycin resistance, respectively. (C, D) FACS profiles of β-gal activity (fluorescein fluorescence) in C2C12 cells expressing only one chimeric receptor, either EGFR–Δα–β-gal (C) or EGFR–Δω–β-gal (D), without EGF treatment (black curve) and treated with EGF for 2 h (red curve). (E) β-gal activity in a population of C2C12 cells expressing both chimeric receptors and treated with EGF for 2 h (red curve). Untreated cells are shown by the black curve. (F) Coexpression of a Fas–Δα–β-gal chimera with the truncated EGFR–Δω–β-gal chimera in NIH-3T3 cells. β-gal activity is shown for cells treated with EGF for 2 h (red curve) or treated with the activating anti-Fas antibody Jo2 for 6 h (100 ng/ml; blue curve). Untreated cells are shown by the green curve. (G) β-gal activity in a clone derived from the cells shown in (E) treated with EGF for 2 h (red curve). Untreated cells are shown by the black curve. (H) FACS profiles of an immunofluorescence assay for the expression of the chimeric receptor in the population of cells shown in (E) (red curve), in the clone shown in (G) (blue curve), and in untransduced cells (green curve)

membrane receptor Fas (CD-95)[14] fused to Δα–β-gal did not yield β-gal activity when either EGF or an activating anti-Fas antibody was added to cells (Fig. 1F). This was not a result of the inability of Fas–β-gal chimeras to dimerize and complement, as Fas receptor activation with the anti-Fas antibody resulted in detectable β-gal activity when Fas–Δα–β-gal and Fas–Δω–β-gal were coexpressed (A.E. and H.M.B., in preparation). These results demonstrate that the complementation of β-gal monitors but does not drive the interaction of the chimeric proteins.

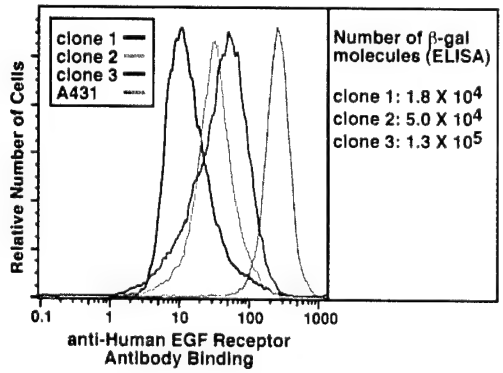

Figure 2. Chimeric receptor expression compared to A431 cells. A431 cells and three clones expressing chimeric receptors were immunofluorescently labeled and assayed by FACS as in Fig. 1H. The same three clones were analyzed by ELISA with an anti-β-gal antibody, and the number of chimeric receptors was determined from a standard curve using purified β-gal protein.

Cells transduced with EGFR–Δα–β-gal and EGFR–Δω–β-gal expressed a broad range of concentrations of the chimeric receptor (Fig. 1H, red curve), which might account for the range in β-gal activity observed in the population. Single cells were isolated using either the FACS or limiting dilution to select for low background amounts of β-gal activity in the absence of EGF and increased amounts of β-gal activity in the presence of EGF. Three dozen clones were tested for ligand-dependent β-gal activity, one of which is shown (Fig. 1G). Clones that had low β-gal activity in the absence of EGF and exhibited a several-fold increase in β-gal activity in the presence of EGF had lower levels of chimeric receptor expression than much of the population (Fig. 1H, blue curve).

We analyzed chimeric receptor expression in three clones that showed ligand-dependent β-gal activity (Fig. 2). Analysis by FACS shows that these clones expressed different amounts of the receptor, but all of them expressed significantly less receptor than the A431 epithelial carcinoma cell line, which is known to express about 2.6×10^6 receptors per cell[15]. The same clones were analyzed by enzyme-linked immunosorbent assay (ELISA) using antibodies to bacterial β-gal and compared to a standard curve generated using purified β-gal protein. These results show that the clones express 1.8×10^5–1.3×10^5 β-gal molecules (chimeric receptors) per cell (Fig. 2), within the range for EGF receptor expression observed in normal cells (up to 3

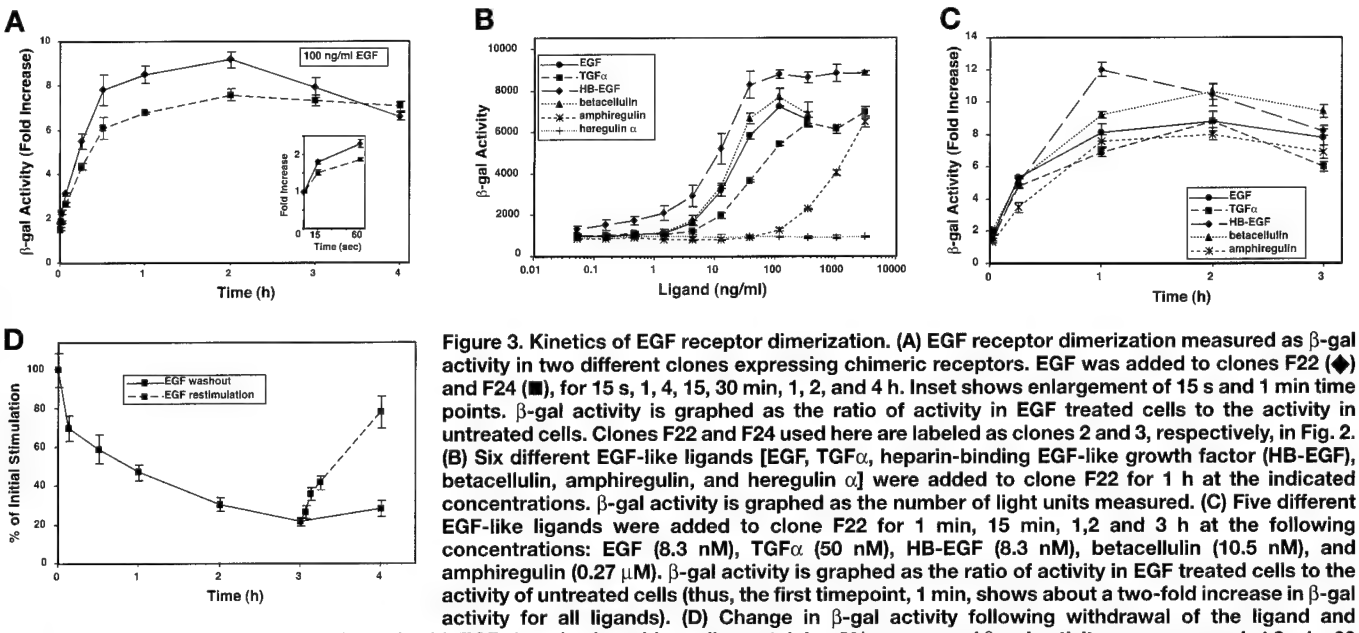

Figure 3. Kinetics of EGF receptor dimerization. (A) EGF receptor dimerization measured as β-gal activity in two different clones expressing chimeric receptors. EGF was added to clones F22 (◆) and F24 (■), for 15 s, 1, 4, 15, 30 min, 1, 2, and 4 h. Inset shows enlargement of 15 s and 1 min time points. β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity in untreated cells. Clones F22 and F24 used here are labeled as clones 2 and 3, respectively, in Fig. 2. (B) Six different EGF-like ligands [EGF, TGFα, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, amphiregulin, and heregulin α] were added to clone F22 for 1 h at the indicated concentrations. β-gal activity is graphed as the number of light units measured. (C) Five different EGF-like ligands were added to clone F22 for 1 min, 15 min, 1,2 and 3 h at the following concentrations: EGF (8.3 nM), TGFα (50 nM), HB-EGF (8.3 nM), betacellulin (10.5 nM), and amphiregulin (0.27 μM). β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity of untreated cells (thus, the first timepoint, 1 min, shows about a two-fold increase in β-gal activity for all ligands). (D) Change in β-gal activity following withdrawal of the ligand and restimulation. Clone F22 was treated for 1 h with EGF, rinsed twice with media containing 2% serum, and β-gal activity was measured at 8 min, 30 min, 1, 2, 3 and 4 h thereafter. After 3 h in the absence of EGF, some samples were again treated with EGF for 4, 8, and 15 min and 1 h. β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity of untreated cells normalized to the value for the initial 1 h treatment which was set to 100%. β-gal activity was measured in triplicate (B and C) or quadruplicate (A and D) samples; error bars indicate standard deviation.

$\times 10^5$ receptors/cells)[16,17]. Thus, β-gal activity is a useful measure of ligand-dependent receptor dimerization without greatly overexpressing the chimeric receptor.

Dimerization kinetics and ligand specificity assayed by β-gal complementation. Although the FACS permits the analysis of live single cells, it is cumbersome to analyze more than 100 samples within a few hours. However, chemiluminescence assays allow rapid analysis of thousands of samples on multiwell culture plates within ~1 h. Using such an assay, we analyzed dimerization kinetics of several ligands at several concentrations. Dimerization, expressed as the fold increase in β-gal activity, could be detected with EGF treatments as short as 15 s (Fig. 3A, inset). With longer exposure to EGF, dimerization continued to increase rapidly for up to 2 h (Fig. 3A). This time course is in good agreement with previous data on EGF receptor kinetics including ligand binding, receptor activation, and substrate phosphorylation, which indicate that the receptor responds to ligand within minutes[18,19].

Six EGF-like growth factors were used to test for the specificity of receptor dimerization and β-gal complementation. The dose/response curves for EGF-like ligands differ (Fig. 3B), but show that dimerization increased with increasing concentrations of the following ligands: EGF, tumor growth factor (TGF)-α, heparin-binding EGF-like growth factor, amphiregulin, and betacellulin. The dose/response curve for amphiregulin demonstrated the significantly lower affinity of amphiregulin relative to the other ligands for the EGF receptor[20]. As expected, dimerization was not induced by the EGF-like growth factor heregulin α, which is known to activate members of the EGF receptor family, but not the EGF receptor itself[20]. The rate of dimerization was measured by β-gal complementation using the concentrations of each ligand found to elicit a maximum response (Fig. 3B) and was similar for all activating ligands (Fig. 3C).

Following removal of the ligand from the medium, EGF receptor dimerization decreased (Fig. 3D). Cells were rinsed to remove free ligand following a 1-h treatment with EGF, at which time point β-gal activity was still increasing (see Fig. 3A). Dimerization began to decline shortly after removal of the ligand. Restimulation of receptor dimerization was induced by the reintroduction of EGF into the medium (Fig. 3D).

Inhibition of receptor dimerization. No significant increase in dimerization was observed in cells treated with both EGF and either monoclonal antibody 225 (Fig. 4) or 528 (not shown), both of which are known to block binding of EGF to the human receptor[21]. Neither an anti-hemagglutinin (anti-HA) antibody or another antibody to the EGF receptor (antibody 455), inhibited EGF receptor dimerization. Monoclonal antibody 13A9 had previously been shown to inhibit TGF-α binding, but not EGF binding, to the receptor[22]. Another study described the surprising finding that although EGF binding and EGF-dependent activation of the EGF receptor occurred in the presence of antibody 13A9, EGF receptor dimerization could not be detected in the presence of this antibody either by immunoprecipitation, by density gradient centrifugation, or by FRET (ref. 23). As expected, we found that antibody 13A9 did inhibit TGF-α induced dimerization. However, in contrast to the previous findings, EGF-stimulated receptor dimerization in the presence of 13A9 was readily detected as β-gal activity (Fig. 4). The same results were obtained with the Fab fragment of anti-

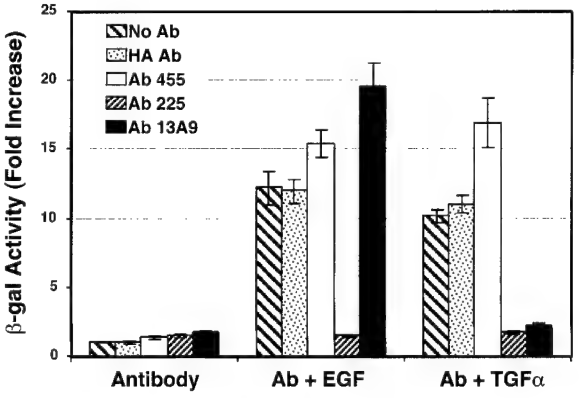

Figure 4. Antibodies which inhibit ligand binding inhibit dimerization. β-gal activity assayed in cells (clone F22) preincubated with monoclonal antibodies (anti-HA and anti-EGF receptor antibody 455, which does not inhibit ligand binding; antibody 225, which inhibits ligand binding; or 13A9, which inhibits TGF-α but not EGF binding) at 25 ng/ml for 5 min and then treated with EGF (100 ng/ml) or TGF-α (300 ng/ml) for 1 h. β-gal activity was measured in quadruplicate samples and is graphed as a ratio of the activity in EGF treated cells relative to untreated cells. Error bars indicate standard deviation.

body 13A9 for both TGF-α and EGF (data not shown).

Because 13A9 does not block receptor activation by EGF, it has been suggested that EGF receptor activation could occur in the absence of dimerization[23]. An alternative explanation presented for this apparent paradox is that 13A9 destabilizes or perturbs receptor dimerization to a degree that prevented detection of dimers by the above methods, but allowed sufficient interaction so that activation occurred[23]. Here we show that receptor dimerization in the presence of antibody 13A9 can in fact be detected if the β-gal complementation assay is used, a result that is in agreement with accepted models in which dimerization precedes receptor activation.

Discussion

Critical to the use of our method for monitoring protein interactions is that the kinetics of the β-gal activity reflect the properties of the interacting non-β-gal portions of the chimeric protein pairs. This appears to be the case based on studies of two different protein pairs. Previously, we showed that in cells expressing chimerae of rapamycin-binding proteins and complementing β-gal mutants, β-gal complementation was specifically induced by addition of rapamycin. β-gal activity began to increase slowly after 30 min, and continued to increase over the subsequent 24h. As shown here, EGF receptor dimerization was detectable as β-gal activity with EGF treatments of as little as 15 seconds and continued to increase, reaching peak levels within one or two hours. Thus, the kinetics of these two distinct sets of interacting proteins monitored by the complementation of the same β-gal mutants differed markedly from one another. Furthermore, β-gal complementation was specific and no enzyme activity was detected if the complementing mutants were linked to non-interacting membrane proteins such as the EGF receptor and Fas. Taken together, these data indicate that β-gal complementation serves to monitor, not to drive, the chimeric protein interactions in each case.

Receptor dimerization has typically been analyzed by in vitro methods such as chemical crosslinking and immunopurification, followed by gel electrophoresis[24,25]. Recently, EGF receptor dimerization has been analyzed in single cells by FRET[13]. Analyses of interactions of proteins conjugated to fluorophores within the cell or cell membrane by FRET have been limited by the difficulty of labeling these molecules in vitro and introducing them at sufficiently high concentrations. This problem has been overcome by the elegant method developed by Tsien and colleagues in which chimeric proteins containing GFP are expressed in cells[5]. By using two different GFP mutants that emit at different wavelengths, FRET analysis of protein interactions is possible[26]. However, unlike the β-gal activity produced by β-gal complementation, the GFP signal cannot be enzymatically amplified. As a result, achieving a detectable signal is limited to some extent by the need to achieve a relatively high level of expression of the chimeric protein.

Since our initial description of the β-gal complementation assay[6], complementation of another protein, DHFR, has been used to monitor protein interactions[7,27]. Complementation of DHFR is assayed by measuring the binding of a fluorescently labeled inhibitor, methotrexate. As with GFP chimeras, the DHFR signal is not enzymatically amplified, and requires a sufficiently high expression level for detection. The DHFR system has been used primarily in cells lacking endogenous DHFR, and has not been used for quantitative assays in mammalian cells that express endogenous DHFR. The enzymatic signal amplification characteristic of the complemented β-gal, as well as the availability of sensitive substrates and rapid assays, enables complementation of this enzyme to be detected without grossly overexpressing the proteins, thus avoiding potential perturbation of normal cellular mechanisms. As shown here, the β-gal complementation system works well without overexpressing the chimeric proteins at high levels, although clearly some dimerization events do not lead to complementation and signal generation (e.g., homodimers between EGF receptors linked to the same β-gal mutant). If a signal were not detected with moderate expression levels, this study would not have been successful as overexpression of the EGF receptor leads to ligand-independent dimerization (Fig. 1). An assay system that does not require overexpression will be invaluable for the study of other proteins such as Fas, as overexpression of Fas can lead to apoptosis.

Because the active bacterial β-gal is known to be a tetramer, it was not obvious that a molecule that requires both complementation and oligomerization to form an active enzyme would be useful in monitoring the dimerization of a linked protein. However, it has been shown that in the formation of an active complemented β-gal protein, the assembly of the complex is relatively rapid, and the rate-limiting step is the conformational changes that occur after oligomerization of the subunits[28,29]. Our data showing that EGF receptor dimerization can be detected with ligand treatments as short as 15 s using β-gal complementation provides support for this model in eukaryotic cells.

Because the cells used here express an endogenous mouse EGF receptor, which can bind ligand in the presence of these human receptor-specific antibodies, the antibody inhibition results show that dimerization of the chimeric human receptor can be blocked when the endogenous receptor is dimerized and activated. Furthermore, evidence that the endogenous receptor is not required for chimeric receptor dimerization is provided by expressing the same constructs in fibroblasts (3T3 clone 2.2), which lack an endogenous mouse EGF receptor but yield similar results to the cells shown here (data not shown). These findings indicate that β-gal complementation is driven by specific interactions of the chimeric receptor, not by a generalized clustering of EGF receptor molecules in the cell.

In conclusion, β-gal complementation provides a rapid and sensitive method for monitoring receptor dimerization at the membrane of live cells. Once the appropriate fusion construct is generated, populations of cells or clone, if necessary, can be obtained and tested within a few weeks. Complementation of β-gal may also provide a screen for novel interacting proteins in a mammalian "two-hybrid" assay in which the protein complexes are monitored in the cell compartment in which they normally occur.

Since complementation occurs in situ in intact live cells, the effect of agents, such as antibody 13A9, on receptor dimerization can be more accurately detected than with previously available methods. This approach provides a potent tool for high throughput screeening for pharmacological agents that can bind to receptors and act as either agonists or antagonists. The blocking antibodies used here have also been shown to inhibit tumor cell growth in vitro and similar reagents that act on the related erbB2 receptor have been used to treat cancer[19,30]. Such potentially therapeutic agents can be rapidly screened using the type of system presented here. That such assays are possible is clear from preliminary results in our laboratory that indicate that β-gal complementation can detect heterodimerization of erbB2 and EGF receptor.

Experimental protocol

Construction and expression of chimeric receptors. The sequence coding for the extracellular and transmembrane domains of the human EGF receptor (amino acids 1–655) was amplified by polymerase chain reaction (PCR). Although this fragment retains Thr654, which is a site of protein kinase C (PKC) phosphorylation, Arg656 and Arg657 are removed, destroying the consensus PKC recognition sequence. The amino acid sequence beginning with Thr654 is Thr-Leu-Glu-Ser-Met, where the methionine is the beginning of the β-gal sequence, and the glutamic acid and serine are generated by the junction sequence and are not native to either EGF or β-gal. The resulting fragment was cloned into the pWZL-Δα and pWZL-Δω vectors, in frame with the Δα and Δω β-gal mutants[6,9]. Virus was produced and cells were

infected as previously described[6,9]. Cells were selected in 1 mg/ml G418 and 1 mg/ml hygromycin, and were maintained in 400 μg/ml of each antibiotic. Populations of cells were ready for analysis within two weeks of infection. Clones were obtained by either cell sorting or limiting dilution. Full-length murine Fas cDNA was similarly cloned into the above vector in frame with the Δα and Δω β-gal mutants.

FACS analysis. Cells were treated with mouse salivary gland EGF (Sigma, St. Louis, MO) at 100 ng/ml. Following all treatments fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was immediately loaded into the cells by hypotonic shock as described[31,32]. Cells were then kept on ice until analysis on the FACS, which occurred 1–2 h after trypsinization. The chimeric receptor was detected by immunofluorescence using a mouse monoclonal antibody specific to the extracellular domain of the human EGF receptor (antibody R1, Dako, Carpinteria, CA, and Calbiochem, La Jolla, CA). Data shown here as FACS profiles in Figure 1 were adjusted for autofluorescence using autofluorescence compensation[33]. Mean fluorescence data for β-gal activity were adjusted for both autofluorescence and endogenous mammalian β-gal activity by subtracting the mean fluorescence of untransduced cells loaded with FDG substrate.

Chemiluminscent analysis of β-gal activity. Triplicate or quadruplicate samples of cells were plated at 10,000/well in 100 μl volume in white 96-well plates (Corning Costar, Acton, MA). After 16–24 h, the spent media was removed and replaced with media containing growth factors, or in some cases, antibodies. Cells were lysed by addition of GAL-Screen substrate (Tropix PE Biosystems, Bedford MA, buffer B formulation), and the plates were incubated at 27°C for 40 min. Luminescence was measured in a Tropix TR717 luminometer, or in a Wallac MicroBeta Plus.

Analysis of kinetics and inhibition of dimerization. Recombinant human growth factors were obtained from Life Technologies, Rockville, MD (EGF), R&D Systems, Minneapolis, MN (TGF-α in Figure 3, heparin-binding EGF-like growth factor, betacellulin, amphiregulin, heregulin α), and Sigma (EGF and TGF-α in Figs 3D and 4). Mouse monoclonal antibodies used were clone 528, clone 225, clone 450, and clone R1 (Calbiochem) and antibody 13A9 (gift of Genentech). Anti-HA mouse monoclonal antibody 12CA5 (Roche Molecular Biochemicals) was used as a negative control. Antibodies were added to cells for 5 min at room temperature before the addition of growth factor, at which point cells were returned to 37°C.

ELISA detection of β-gal chimeric proteins. Cells were lysed in lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.01% thimerosol, and a protease inhibitor cocktail containing AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma)] and lysates were centrifuged to remove debris. ELISA plates were coated with 1 μg/ml mouse anti-β-gal antibody 5B88 (Life Technologies) in 4 μg/ml BSA in PBS and then blocked for 2 h (Superblock, Pierce Chemical, Rockford, IL). Cell lysates were diluted in lysis buffer with 0.2% BSA and 0.05% Tween-20 and 100 μl of diluted lysate representing 1×10^4 to 1×10^5 cells was added to each well. To generate a standard curve, purified *Escherichia coli* β-gal protein (Sigma) was diluted in the same buffer to final concentrations of 1.2 pg–20 ng per well. After 30 min, wells were washed five times with 0.2% Tween-20 in PBS. To each well were added 100 μl of biotinylated mouse anti-β-gal antibody GAL-13 (Sigma) diluted 1:5,000 in 0.2% BSA, 0.05% Tween-20 in PBS; these were incubated for 30 min at room temperature, and then washed as before. To each well were then added 100 μl of streptavidin–alkaline phosphate conjugate (Tropix) diluted 1:5,000 in 0.2% BSA, 0.05% Tween-20 in PBS; these were incubated for 25 min at room temperature, and then washed as before. Finally, 100 μl of CSPD-Sapphire II chemiluminescent substrate (Tropix) were added to each well, and after 30 min, the luminescence was measured on a Tropix TR717 luminometer.

Acknowledgments

We thank Xiao-Jia Chang, Carol Zuschlag, Carol Charlton, Joyce Tung (funded by Howard Hughes Medical Institute undergraduate research fellowship), and Patricia Wong (funded by the Stanford Medical School Alumni Scholarship) for technical assistance. F.M.V.R. was funded by a fellowship from the Human Frontier Science Program. This research was supported by National Institute of Health grants to H.M.B.

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Protein–Protein Interactions

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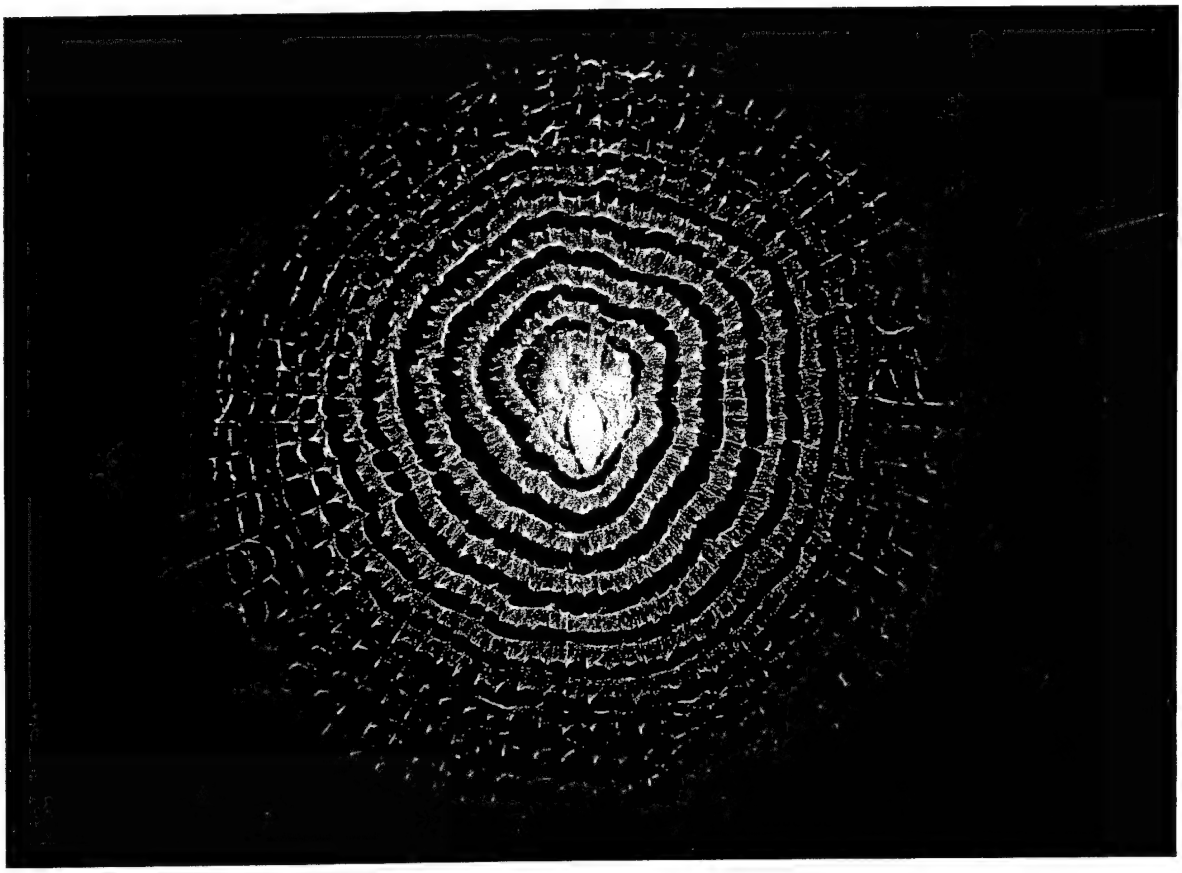

Edited by

Erica Golemis

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COLD SPRING HARBOR LABORATORY PRESS
Cold Spring Harbor, New York, 2002

23 Protein Interactions in Live Cells Monitored by β-Galactosidase Complementation

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INTRODUCTION

The characterization of protein interactions is important to the understanding of signal transduction pathways and cellular processes. Here we describe a method utilizing β-galactosidase (β-gal) complementation that can monitor protein interactions in live mammalian cells (Rossi et al. 1997, 2000; Blakely et al. 2000). In brief, the method involves expressing chimeric proteins consisting of two potentially interacting proteins of interest fused to complementing β-gal deletion mutants. When the two proteins of interest interact, the β-gal mutants complement, reconstituting an active β-gal enzyme. Some of the advantages of β-gal complementation are: (1) It is a direct assay, that is, a signal is generated at the site of the interaction, and activation of a reporter gene is not required; (2) it is a sensitive assay, due to enzymatic amplification of the product; (3) over-

expression is not required—interactions can be detected at physiological levels of expression; (4) β-gal enzyme activity can be measured using a variety of substrates that permit quantitative assays that can measure β-gal activity in single live cells, or that are amenable to high-throughput screening technologies.

A variety of biochemical methods is available for demonstrating the interaction of two proteins, the most common of which is immunoprecipitation followed by identification by immunoblotting. Such biochemical methods have proven reliable, but can be limited by the inability of some complexes to survive cellular disruption and immunoprecipitation. Complexes can be stabilized by chemical cross-linking before the cell is disrupted, but this step can introduce artifacts by cross-linking adjacent, but noninteracting, proteins. The yeast two-hybrid system has been the most powerful method for identifying novel protein interactions (Fields and Song 1989; Bai and Elledge 1996). However, proteins in this system must be capable of interacting in the yeast environment and must then translocate to the nucleus and activate transcription of a reporter gene.

An ideal system for studying protein interactions would generate a signal upon the interaction of the proteins of interest. This signal would be specific, and would be detectable in the cellular compartment in which it is generated, either directly or with minimal additional steps, such as addition of a substrate. Ideally, the signal would be detected in live cells, or the signal must be able to survive disruption of the cells. Several recent developments have come close to this goal. Fluorescence resonance energy transfer (FRET) has been used to study protein interactions via the in vitro labeling of two proteins of interest with fluorescent tags (Adams et al. 1991; Gadella and Jovin 1995; Chapter 10). However, the difficulties of introducing fluorescently labeled proteins into cells at sufficiently high concentrations to detect a signal can limit the utility of this method. An alternative FRET methodology involves expressing chimeric proteins that incorporate one partner of an interacting protein pair and a fluorescent protein, such as green fluorescent protein (GFP). By using two different GFPs, FRET analysis of the interacting chimeras is possible, but it remains to be seen whether this method will work well with a variety of interacting proteins (Miyawaki et al. 1997; Pollok and Heim 1999). Other non-FRET methods using interacting chimeric proteins depend on the complementation of two mutant protein fragments to reconstitute a functional protein. Complementation of dihydrofolate reductase (DHFR) has been used to examine protein interactions in mammalian cells, but this system is dependent on either a nonenzymatic substrate-binding assay that is quantitative only in cell lines that lack endogenous DHFR, or a nonquantitative enzymatic survival assay that only works in cells lacking endogenous DHFR (Pelletier et al. 1998; Remy et al. 1999). The complementation of bacterial β-gal, discussed in detail below, generates an enzymatically amplified signal that can be detected quantitatively by a variety of assays in many different cell types without overexpression of the proteins (Rossi et al. 1997, 2000; Blakely et al. 2000).

BACKGROUND

Intracistronic β-gal complementation is a phenomenon first observed by Jacob and Monod, in which two mutants of the bacterial enzyme β-gal that have inactivating deletions in different critical domains recreate an active enzyme by sharing the intact domains (Ullmann et al. 1965, 1967). β-Gal complementation has been used for decades as a marker for molecular cloning experiments in bacteria (blue-white colony selection). The basis of this system is the expression of a truncated inactive β-gal protein (ΔM15) by the host bacterium, which can be complemented by expression of a peptide (α peptide) from a plasmid cloning vector, but only if the α-peptide coding sequence is not disrupted by a cloned cDNA. β-gal was successfully complemented in mammalian cells using three different complementing peptides (Fig. 1): Δα, similar to the ΔM15 mutant used in bacteria, has a deletion near the amino terminus of the protein (amino acids 11–41); Δω, trun-

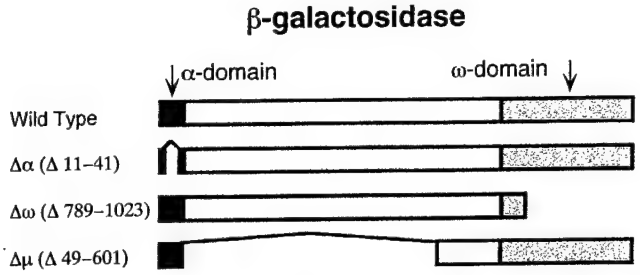

FIGURE 1. Schematic diagram of the β-gal deletion mutants. The α and ω domains, as defined by Jacobson et al. (1994), are represented by the black and gray boxes at the amino and carboxyl termini of the protein, respectively. Δα has a small deletion in the α domain, Δω has a large deletion of the carboxyl terminus, and Δμ has a large deletion in the region of the protein between the α and ω domains.

cated at amino acid 788, lacks the carboxyl terminus of the protein; and Δμ has a deletion (amino acids 49–601) in the middle of the protein (Mohler and Blau 1996). The amino-terminal (α) and carboxy-terminal (ω) regions of the protein are critical to forming a functional enzyme (Villarejo et al. 1972; Jacobson et al. 1994). Δω functions as an "α donor" (analogous to but much longer than the α peptide used in bacterial complementation), Δα functions as an "ω donor," and Δμ can provide either region but lacks the middle region that is important for the structure of the active enzyme. Any two of these mutant proteins, when expressed in the same cell, result in formation of an active β-gal enzyme (Mohler and Blau 1996). Although native β-gal forms a functional enzyme as a homotetramer, it has not been definitively established whether the active complemented enzyme complex in mammalian cells requires eight mutant proteins.

β-Gal complementation in mammalian cells was first used to study the process of cell fusion in myoblast differentiation (Mohler and Blau 1996). Each of the deletion mutants was expressed in separate populations of myoblasts. When two of the different populations were cocultured in differentiation-inducing conditions, the myoblasts fused into multinucleated syncytia, or myotubes, and complementation of β-gal was observed. Measurement of β-gal activity provided a simple quantitative assay for myoblast fusion and thus provided a rapid method to measure the effects of genetic mutations and culture conditions of myoblast differentiation (Charlton et al. 1997).

By constructing chimeric proteins incorporating one of the β-gal deletion mutants, interactions between the non-β-gal components of the chimeras can be detected (Rossi et al. 1997). Coexpression of any two of the three different deletion mutants in the same cell results in β-gal complementation, but lower activity was usually observed when Δα was paired with Δω, compared to either of these mutants with Δμ (Mohler and Blau 1996). In a complementation assay for protein interactions, the interaction should be driven by the proteins of interest, rather than the β-gal mutants; therefore, the weakest complementing pair, Δα and Δω, were used for constructing chimeras for protein interaction assays. In the initial test of this system, Δα and Δω were linked to the rapamycin-binding proteins FRAP and FKBP12 (Rossi et al. 1997). β-Gal activity was very low in the absence of rapamycin but increased significantly upon addition of rapamycin. β-Gal activity was dependent on the dose of rapamycin and continued to increase throughout the time measured (Fig. 2, left).

Membrane receptor dimerization can also be quantitated using β-gal complementation (Blakely et al. 2000). By expressing chimeric proteins that link Δα or Δω to a truncated epidermal growth factor (EGF) receptor, EGF-induced receptor dimerization resulted in increased β-gal activity. β-Gal activity initially increased more rapidly than was observed with rapamycin-binding proteins; however, after increasing about eightfold in the first hour, the activity plateaued, as would be expected with membrane receptor dimerization (Fig. 2, right). Thus, the kinetics of β-

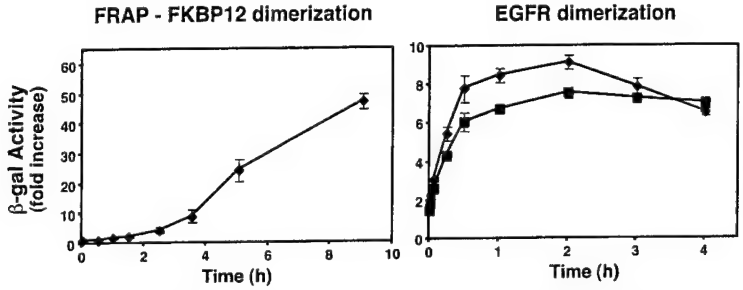

FIGURE 2. The kinetics of chimeric β-gal protein complementation depend on the non-β-gal portion of the chimera. The rapamycin-binding proteins FRAP and FKBP12, or the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR), are fused to β-gal deletion mutants and expressed in C2C12 cells. The left panel shows that β-gal activity increases over a 9-hour period following the addition of rapamycin to a population of cells expressing the FRAP and FKBP12–β-gal chimeras. The right panel shows that β-gal activity increases rapidly over the first 1–2 hours and then plateaus after the addition of EGF to two different clones expressing the EGFR–β-gal chimeras. The β-gal activity in each experiment reflects the dimerization kinetics of the wild-type rapamycin-binding proteins and EGFR, respectively.

gal activity reflect the kinetics of the non-β-gal components of the chimeric proteins. β-Gal complementation was used to characterize fully the effects of an anti-EGF receptor antibody on receptor dimerization, which previously had not been possible using biochemical methods. Furthermore, the levels of expression of the chimeric receptors used in these studies did not exceed the levels of the endogenous receptor in normal cells.

Finally, β-gal complementation, in addition to measuring cytoplasmic protein interactions and membrane protein interactions, can also measure the interaction of a cytoplasmic protein and a membrane receptor (e.g., TGF-β receptor 1 and FKBP12; T.S. Wehrman et al., unpubl.). Thus, β-gal complementation can measure three types of interactions that are key components of most signal transduction pathways (Fig. 3).

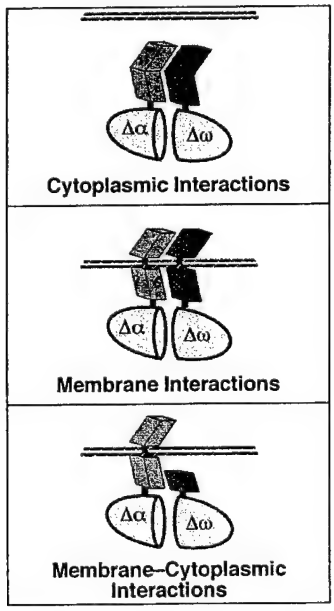

FIGURE 3. β-Gal complementation can detect protein interactions in the cytoplasm or at the membrane. Two chimeric proteins containing different β-gal deletion mutants (labeled Δα or Δω) and proteins of interest (shaded rectangular boxes in figure) are expressed in cells. If the proteins of interest interact, the β-gal mutants will complement, and β-gal enzyme activity serves as a measure of the interaction.

β-Gal complementation can be used to confirm protein interactions and to characterize known interactions. A variety of assays is available to measure β-gal activity, further increasing the utility of this method. Of particular interest are a flow cytometry assay using a fluorogenic substrate for β-gal for the measurement of β-gal activity in single live cells and a chemiluminescent assay that enables the analysis of hundreds of samples in microtiter plates using high-throughput screening technologies. The simple, rapid assays available for β-gal make it possible to test the effect of a variety of conditions (e.g., inducers, inhibitors, concentrations, antibodies, cell backgrounds). β-Gal complementation will also be useful for screening for novel inducers and inhibitors of protein interactions using high-throughput screening methods (e.g., receptor agonists and antagonists) and potentially could be used as a "mammalian two-hybrid" screen for novel interactions.

OUTLINE OF PROCEDURE

The first step is constructing two chimeras each containing one of the β-gal deletion mutants and the protein of interest. These constructs must then be expressed in cells. We use retroviral vectors to express the chimeras to rapidly obtain cells that stably express the constructs while limiting the copy number of the construct and thus the expression levels. Once cells that express both constructs are obtained, the cells can be tested for β-gal activity in the presence and absence of an inducer of the desired interaction. In some cases, cloning the cells that express the chimeras may be desirable. Several different assays for β-gal activity are presented here.

Strategy for Construction and Expression of the Chimeric Proteins

Although our initial experiments were carried out with the β-gal deletion mutant linked to the carboxyl terminus of the protein of interest, we have subsequently found that, at least in the case of cytoplasmic rapamycin-binding proteins, complementation works equally well with the β-gal mutants at either end of the chimeric protein (T.S. Wehrman et al., unpubl.). In the case of membrane proteins, the β-gal mutant has only been placed at the carboxyl terminus, so that complementation occurs in the cytoplasm. The use of PCR and standard cloning techniques is required to place the protein of interest accurately in frame with the β-gal deletion mutant.

The use of retroviral vectors simplifies the expression of the chimeric proteins. We use two retroviral vectors, which contain either Δα or Δω under the transcriptional control of the viral long terminal repeat (LTR) (Fig. 4) (Rossi et al. 1997; Blakely et al. 2000). An intracistronic ribosome entry site (IRES) and a drug-resistance marker are located downstream of the β-gal mutant. After the protein of interest is cloned in frame with the deletion mutant, the plasmid containing the retroviral vector is transiently transfected into the Phoenix-E packaging cell line using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Indiana) according to the manufacturer's instructions. Although calcium phosphate transfection protocols can sometimes result in transfection of a higher percentage of the cells, we consistently obtain higher viral titers using FuGENE 6, possibly because of the absence of toxic side effects. Virus supernatant is used immediately to infect the target cells, or it can be frozen at –70°C with a resulting small drop in titer. Although many cell lines can be infected with nearly 100% efficiency, any uninfected cells can be eliminated through the use of the selectable marker in the vector.

Although one can infect cells with both vectors (the Δα and Δω constructs) simultaneously, we usually infect the cells sequentially. It can be useful to have cells on hand expressing only one of the vectors in case future experiments call for changing one of the interacting proteins. Furthermore, if there are problems with expression of the chimeric proteins, it may be easier to determine which of the constructs is defective if they are singly expressed, especially if the two

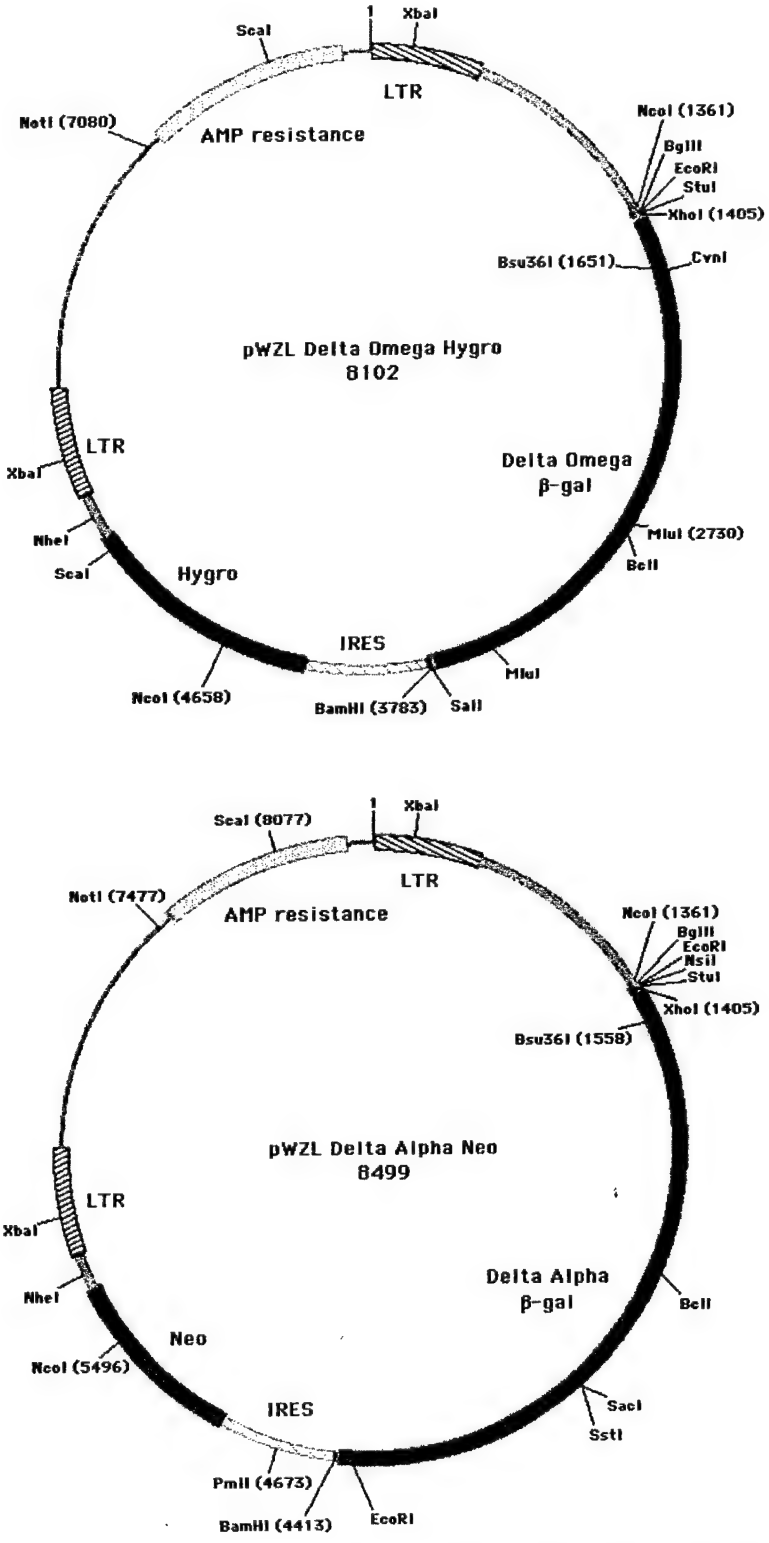

FIGURE 4. Maps of the β-gal complementation vectors. Using the plasmids shown, chimeric proteins can be engineered by using PCR and restriction digests to clone cDNAs of interest in frame with either the Δα or Δω mutants of bacterial β-gal. When transfected into packaging cell lines, retroviruses are produced (lacking the ampicillin-resistance gene and other bacterial sequences between the LTRs) that express the chimeric protein and a selectable marker (resistance to G418 or hygromycin).

chimeric proteins are similar in size. Selection with antibiotics such as G418 and hygromycin should be carried out at the lowest dose that effectively kills uninfected cells, because selection with higher doses can lead to overexpression of the constructs.

Assays for β-Gal

Chemiluminescent Assay for β-Gal

The chemiluminescent assay for β-gal is the quickest and simplest method for assaying a large number of samples. In brief, cells are plated and treated with reagents that will induce the protein interaction of interest. Gal-Screen reagent is added to the cells, resulting in the prolonged production of photons ("glow" kinetics, not "flash" kinetics) in the presence of active β-gal. The light emitted by the sample is then measured in a luminometer. Cells are plated on a 96-well plate, a format that permits the use of high-throughput screening instrumentation if desired. This format greatly simplifies the assay because cells are plated and assayed on the same dish. However, if a microplate luminometer is not available, the samples can be transferred to a tube for use in a tube luminometer.

The microtiter plate used in this assay should be of the type recommended by the manufacturer of the luminometer. For many instruments, this will be a white plate with either a white (opaque) or clear bottom. However, some instruments may specify the use of black plates. Although opaque plates are ideal, clear-bottomed plates, which permit observation under a microscope, can be used until the experimenter is comfortable that he is obtaining a subconfluent, evenly plated culture. Chemiluminescence can spill from one well to another through the clear bottom of the plate, which will be noticeable if a well producing thousands of induced light units is adjacent to a negative control or uninduced sample.

We use a Tropix TR717 microplate luminometer, although other instruments also work well, including the following instruments that we have tested: EG&G Wallac Berthold LB96V (which is nearly identical to the Tropix instrument), Turner Designs Reporter, and the EG&G Wallac MicroBeta Plus (current version is MicroBeta Trilux; MicroBeta units with the automatic injector may have reduced sensitivity). One disadvantage of the MicroBeta is that it takes 5–10 minutes to count a plate, compared to less than 2 minutes for the others. Some instruments may not have sufficient sensitivity, and this should be considered if β-gal activity is not detected.

Flow Cytometry Assay for β-Gal

By assaying the product of the fluorogenic β-gal substrate fluorescein di-β-D-galactopyranoside (FdG) using a flow cytometer, β-gal activity can be measured in single live cells. This is useful for determining changes in β-gal activity in a population of cells and thus determining whether or not cloning of the cells is necessary to achieve a uniform response. Furthermore, like the chemiluminescent assay, the FdG assay is quantitative and can be used to study the kinetics and dose response of the interaction. The fluorescence-activated cell sorter (FACS) can also be used to isolate subpopulations of cells or to clone cells that exhibit the desired response.

The protocol given here is based on that published by Nolan et al. (1988) and has been modified largely to accommodate the processing of large numbers of samples. Using this method, one person can readily assay 50–100 samples, and with two persons working together, as many as 200 samples can be assayed within a few hours. If only a few samples are to be analyzed, the assay can be completed in less than half an hour. The FdG substrate used in this assay is not cell-permeable and must be introduced into the cells by a hypotonic shock. Cleavage by active β-gal produces free fluorescein, which is also unable to cross the plasma membrane and remains inside the cells that have complemented β-gal. Following the analysis, most cells remain viable and can be returned to culture. In populations or clones that exhibit homogeneous changes in β-gal activity, the mean fluorescence of each sample is a reliable indicator of the interaction.

Protocol 1

Preparation of Retrovirus and Infection of Target Cells

Retroviral vectors are used here to express the chimeras and obtain cells that express the constructs.

MATERIALS

Buffers and Reagents

Dulbecco's modified Eagle medium (DMEM), high glucose formulation (450 g/liter)
Fetal bovine serum (FBS; HyClone)
Polybrene (8 mg/ml) (1000x) in H_2O, filter-sterilized (Sigma)

Vectors

FuGENE 6 (Roche Molecular Biochemicals)

The retroviral vector should not exceed 9 kb in length to ensure efficient packaging of the vector. This limit does not include the portions of the plasmid required for plasmid propagation which are not incorporated into the retrovirus.

Plasmids and Cells

Phoenix-E cells

For information on obtaining these cells, see Dr. Garry Nolan's Web site at http://www.stanford.edu/group/nolan/mtas.html).

Plasmids containing β-gal deletion mutants fused in frame to the protein of interest

Special Equipment

Benchtop centrifuge with microplate carriers

METHOD

1. Transfect the plasmid containing the retroviral construct into the Phoenix-E packaging cell line using FuGENE 6. (The procedure is outlined here, but it is strongly recommended that the manufacturer's instructions be followed.)

   a. Plate 1.5×10^6 to 2×10^6 Phoenix-E cells per 60-mm dish in DMEM containing 450 g/liter glucose (high glucose formulation) and 10% FBS the day before transfection (or 3×10^6 cells on the day of transfection). Cells should be 50–80% confluent at transfection.

   b. Mix the following components in a microcentrifuge tube in the order given: serum-free DMEM, 6 µl of FuGENE 6 (vortex stock tube first, then add reagent directly to the medi-

X-Gal and Fluor-X-Gal Assays for β-Gal

The X-gal assay is a simple chromogenic assay that requires no specialized instrumentation. In the presence of active β-gal, the substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and a potassium ferricyanide buffer, a blue precipitate that can be observed by microscopy forms in fixed cells. This assay is not quantitative and is not particularly sensitive, but it can be used to detect β-gal complementation if the induction of complementation is sufficiently robust (greater than about fivefold) (Rossi et al. 1997).

Because the X-gal assay product quenches fluorescence, the Fluor-X-gal assay was developed so that β-gal activity could be assayed in cells that had been immunofluorescently labeled for other proteins (Mohler and Blau 1996). This assay combines an azo dye, Fast Red Violet LB, with either X-gal or 5-bromo-6-chloro-3-indolyl β-D-galactopyranoside (5-6 X-gal) to form a fluorescent precipitate in the presence of active β-gal. Fluor-X-gal is more sensitive than X-gal, but like X-gal, is not quantitative and requires fixation of the cells.

Protocol 2

Chemiluminescent Assay for β-Gal

This protocol is the quickest and simplest method for assaying a large number of samples.

MATERIALS

Buffers and Reagents

Cells from Protocol 1
Gal-Screen reagent (Tropix)
Phosphate-buffered saline (PBS)

Special Equipment

Microplate luminometer
Plates (96-well) appropriate for the microplate luminometer used

METHOD

1. Plate cells in 96-well plates the day before the assay at a density of 10,000 cells per well (number established for C2C12 or C2F3 mouse myoblasts) in a volume of 100 µl in medium appropriate for the cell line used. Cells should be subconfluent at the time of the assay.
2. Treat cells with reagents appropriate to induce or modify the interaction under study. Typically, each treatment is carried out on triplicate or quadruplicate wells.
3. At the end of the treatment period, aspirate the medium from the wells and add 200 µl of Gal-Screen reagent prepared according to the manufacturer's instructions (substrate is diluted 1:25 immediately before use with Gal-Screen buffer B equilibrated to room temperature) but additionally diluted 1:1 with 1x PBS.

   Removing the medium is not recommended by the manufacturer's instructions, but this is necessary if, because of kinetic analysis, some of the wells were refed with fresh medium at different time points. Removal of the medium also leads to a slight improvement in the signal-to-noise ratio. The Gal-Screen reagent is additionally diluted 1:1 with PBS to compensate for the removal of the medium from the plate.
4. Incubate the plate for 45 minutes to 1 hour at 26–28°C. Alternatively, incubate at room temperature for a period of time sufficient for the Gal-Screen reaction to reach a plateau.
5. Read the plate using a microplate luminometer, measuring each well for 1 second or for a time appropriate for the instrument used.
6. Analyze data using appropriate software.

um, not to the side of the tube), 2 μg of plasmid DNA. The total volume should be 100 μl. Mix the components by gently flicking the tube; do not vortex the tube. Incubate at room temperature for 15–30 minutes. The volumes can be scaled for multiple plates receiving the same plasmid, or for different-sized plates.

c. Add the 100-μl FuGENE–DNA mixture dropwise to the plate of Phoenix-E cells. It is not necessary to change the medium first or to use serum-free medium on the cells.

2. Incubate overnight (18–24 hours), and then refeed the cells with 2–4 ml of DME (high glucose) + 10% FBS.

3. At least 30 hours after transfection and at least 6 hours after refeeding, remove the medium (viral supernatant) from the dish and filter through a 0.45-μm syringe filter.

4. Add Polybrene to a final concentration of 8 μg/ml to the viral supernatant, or immediately freeze the viral supernatant at –80°C.

5. Aspirate medium from a subconfluent dish of target cells and replace with sufficient viral supernatant containing Polybrene to cover the surface of the dish. If the target cells cannot survive in the Phoenix-E media, dilute the Phoenix-E media 1:1 or more in target cell media.

6. *Optional:* To increase viral infection efficiency, centrifuge the plates (up to 100 mm in diameter) containing the target cells in a benchtop centrifuge equipped with microplate platforms at 2500 rpm (Beckman GS-6 centrifuge or equivalent) for 30 minutes.

   Place dishes carefully in the center of the microplate carrier and make sure that the rotor is balanced. Sealing the plate with Parafilm will help the dish "stick" to the center of the carrier.

7. *Optional:* Continue to harvest retrovirus from the producer cells every 6–12 hours up to 72 hours after transfection, and then discard them. Either use the harvested supernatant for additional rounds of infection or freeze immediately at –80°C.

8. Refeed the target cells 6 hours after adding retrovirus, or, to increase infection efficiency, add another aliquot of viral supernatant containing Polybrene as above. A third round of infection may be carried out after another 6 hours if desired.

   Although centrifugation and multiple rounds of infection can increase the infection frequency, it is not desirable to have multiple copies of the retrovirus in a cell, as this can lead to overexpression. To ensure single-copy transduction in most of the cells, an infection efficiency of 15% is desired. Thus, the viral supernatant may have to be diluted. Infection efficiency can be measured using any retroviral vector expressing β-gal, GFP, or any readily observable marker. However, many of the experiments described here were accomplished without precise titration of the virus.

9. Refeed the target cells with medium containing the appropriate antibiotic to select for infected cells 24 hours after the final round of infection. The time required for uninfected cells to die depends on the cell line used. Cells should continue to be cultured in the selective antibiotics throughout the experiment to prevent a loss of expression of the chimeric proteins.

Protocol 3

Flow Cytometry Assay for β-Gal

Flow cytometric assay of FdG measures β-gal activity in live cells so that a determination can be made of whether or not cloning of the cells is needed. In addition, the FdG assay is quantitative and can be used to study the kinetics and dose response of the interaction.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

Dimethylsulfoxide (Sigma) <!>
Fluorescein di-β-D-galactopyranoside (FdG, Molecular Probes) <!>
Phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS)
Propidium iodide (10 mg/ml in H_2O) (Sigma) <!>

Special Equipment

Benchtop centrifuge with adapters for holding 5-ml tubes
Flow cytometer
Pipetman P-1000
Polystyrene tubes (5-ml round-bottomed; Falcon 2058)

METHOD

1. Plate cells in appropriate medium the day before the assay in 24-well plates at a density such that the culture will be subconfluent at the time of the assay (50,000 cells per well in 0.5 ml of medium for C2C12 or C2F3 mouse myoblasts).
2. Treat cells with reagents appropriate to induce or modify the interaction under study. Typically, different treatments are carried out on duplicate or triplicate samples.
3. At the end of the treatment period, aspirate the medium from the wells and rinse once with PBS.
4. Add two drops of trypsin solution and incubate (room temperature or 37°C) for 5 minutes or until a firm tap on the side of the dish dislodges most cells.
5. Add 1 ml of PBS + 5% FBS to each well. Using a Pipetman P-1000 or equivalent, triturate the cell suspension and rinse the bottom of the well to remove all cells. Transfer cell suspension to 5-ml clear polystyrene tubes (Falcon 2058) appropriate for use on a flow cytometer.

6. Pellet cells by spinning in a benchtop centrifuge at about 1500 rpm for 5 minutes.
7. Remove the supernatant by inverting tubes (if possible, invert entire centrifuge bucket adapter assembly with the tubes still in it). While tubes are still inverted, aspirate the remaining drop of liquid from the lip of each tube.

   Beckman sells an insert that can be added to their adapter for 5-ml tubes that prevents the tubes from falling out when the adapter is inverted.
8. Add 100 µl of PBS + 5% FBS (room temperature) to each tube. Vortex to resuspend the cells (vortex the entire centrifuge bucket adapter assembly with tubes).
9. Prepare a 100x stock of substrate by adding 100 µl of dimethylsulfoxide to a 100-µg vial of FdG. Dilute appropriate amount of substrate in sterile deionized H_2O.

   After preparation, the stock can be stored for several weeks at –20°C protected from light.
10. Add 100 µl of substrate to each tube of cells (hypotonic shock). Incubate for 3 minutes at room temperature.
11. Stop the uptake of FdG by adding 2.0–2.5 ml of ice-cold PBS + 5% FBS + 1 µg/ml propidium iodide to each tube. Pellet cells at about 1500 rpm for 5 minutes.

    Propidium iodide is a fluorescent compound that accumulates in dead cells. Thus, cells with propidium iodide fluorescence can be excluded from the data analysis and sorting.
12. Remove most of the supernatant by gently inverting the tubes. If possible, invert the entire centrifuge bucket adapter assembly with tubes still in it. DO NOT shake tubes or allow liquid that clings to lip of tube to come out. Return the tubes to an upright position and vortex the entire centrifuge bucket adapter assembly with tubes. Sufficient liquid should have been retained to resuspend the cells in a volume appropriate for use on the flow cytometer (about 200 µl).
13. Place tubes on ice. Analyze the cells by flow cytometry.

Protocol 4

X-Gal Assay for β-Gal

This assay, which is not quantitative or especially sensitive, can be used to detect β-gal complementation if the induction of complementation is sufficiently robust.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

Dimethylformamide (Sigma)
Magnesium chloride ($MgCl_2$) <!>
Paraformaldehyde (4%) <!> in PBS
Potassium ferricyanide ($K_3Fe(CN)_6$) <!>
Potassium ferrocyanide ($K_4Fe(CN)_6$) <!>
X-Gal (Sigma)

METHOD

1. Plate the cells on tissue culture dishes or on glass coverslips at subconfluent densities.
2. Treat the cells with reagents appropriate to induce or modify the interaction under study.
3. Fix the cells for 4 minutes with cold (4°C) 4% paraformaldehyde in PBS. Rinse with PBS twice for 5 minutes.
4. Prepare the substrate by diluting a stock solution of X-gal (40 mg/ml in dimethylformamide, stored at –20°C protected from light) to a final concentration of 1 mg/ml in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS.
5. Add the diluted X-gal to cells (sufficient volume to cover cells). Incubate overnight at 37°C (shorter times are sufficient for high levels of β-gal activity; monitor the reaction under a microscope).
6. Examine the dish or coverslip microscopically for blue cells.

Protocol 5

Fluor-X-gal Assay for β-Gal

This assay determines β-gal activity in cells that were immunofluorescently labeled for other proteins.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

4′,6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) <!>
Fast Red Violet LB (Sigma)
Paraformaldehyde (4%) <!> in PBS
5-6 X-gal (Fluka)

Special Equipment

Epifluorescence microscope

METHOD

1. Plate the cells on glass coverslips at subconfluent densities.
2. Treat the cells with reagents appropriate to induce or modify the interaction under study.
3. Fix the cells for 4 minutes with cold (4°C) 4% paraformaldehyde in PBS. Rinse with PBS twice for 5 minutes.
4. If cells are to be immunofluorescently labeled with antibody as well as Fluor X-gal, carry out all immunolabeling procedures at this point at 4°C.
5. Prepare the reagent by diluting into PBS a stock solution of Fast Red Violet LB (50 mg/ml in dimethylformamide and store at –20°C. The substrate will not completely dissolve at this concentration) to a final concentration of 100 μg/ml, and a stock solution of 5-6 X-gal (50 mg/ml in dimethylformamide; store at –20°C. The solution will change from pale blue to yellow after exposure to light, but this does not appear to affect activity) to a final concentration of up to 25 μg/ml (decrease the concentration of 5-6 X-gal if β-gal activity is strong). Filter through a 0.45-μm syringe filter to remove any precipitate.
6. Add the mixture of diluted Fast Red Violet LB and 5-6 X-gal to cells (sufficient volume to cover cells). Incubate for 60–90 minutes at 37°C.
7. Rinse in PBS for 30 minutes at room temperature.
8. *Optional:* Nuclei may be stained by diluting DAPI in PBS to a final concentration of 100 ng/ml and incubating cells for 10 minutes at room temperature, followed by two rinses in PBS for 5 minutes.
9. Mount the coverslips in PBS and seal with nail polish.
10. Detect Fluor X-gal staining with either the fluorescein (FITC) or rhodamine (TRITC) filter sets of an epifluorescence microscope. The FITC channel gives a better signal-to-background ratio for weak signals, but strong signals appear to be quenched. Therefore, Fluor X-gal stain is best viewed with TRITC filters.

TROUBLESHOOTING

In some experiments, the initial population of cells expressing the chimeric constructs will have low β-gal activity in the absence of the interaction of interest and will exhibit a severalfold increase in β-gal activity when an inducer of the interaction is present. However, in other experiments, no induction or very poor induction may be observed. Low β-gal activity accompanied by a lack of induction can be caused by a failure of one of the constructs to properly express the chimeric protein. Constitutive β-gal activity can be caused by overexpression of one or both of the constructs.

No β-gal Activity Is Observed

If no β-gal activity is observed, the expression of the chimeric constructs must be confirmed. This is best accomplished by immunoblotting cell extracts using antibodies to either β-gal or the non-β-gal component of the chimera. If an immunoreactive protein of the appropriate size is observed, the chimera is being expressed. No immunoreactive band indicates a lack of expression, and a band that is too small indicates that the protein is likely terminated at the wrong residue. Such errors can often be identified by sequencing the cDNA for the chimeric protein as well. If antibodies for immunoblotting are not available for the non-β-gal component of the chimera, commercial antibodies for β-gal are available. We have had mixed success at immunoblotting β-gal chimeras expressed in mammalian cells using anti-β-gal antibodies. The best immunoblots have been obtained using a cocktail of the following antibodies: Calbiochem (La Jolla, California) anti-β-gal monoclonal antibody (OB02-100); Sigma (St. Louis, Missouri) anti-β-gal polyclonal antisera (G4644); Sigma anti-β-gal monoclonal (G6282); each was diluted 1:1000. However, if good antibodies to the non-β-gal protein are not available, we recommend adding a peptide tag to the chimeric protein.

Constitutive β-gal Activity Is Observed

Inducible interactions will ideally have low β-gal activity in the absence of the inducer or ligand. If constitutive β-gal activity is observed, several steps can be taken to reduce the uninduced levels of β-gal activity. First, it is important to avoid overexpressing the β-gal constructs. One significant advantage of this system is that the signal is enzymatically amplified and therefore the constructs do not have to be overexpressed (Blakely et al. 2000). Overexpression can result from selection at excessively high concentrations of drug. For example, we have observed higher levels of expression in C2C12 cells selected at 1 mg/ml of G418 or hygromycin rather than at 300 μg/ml, which is the minimum amount of drug required for selection in this cell line. The use of retroviral vectors limits the number of copies of the construct to a few per cell, which can also help to avoid overexpression. To ensure that most cells receive only a single copy, it is necessary to titrate the virus so that only 15% of cells are infected, according to the Poisson distribution.

In cases where there is a high level of uninduced β-gal activity or poor induction (less than a severalfold increase) of β-gal activity, it is helpful to examine the population of cells on a flow cytometer by assaying for β-gal activity using the fluorogenic substrate FdG (Blakely et al. 2000). If uninduced β-gal activity in the population is not uniform (both high- and low-activity cells are observed), a subpopulation or clones of inducible cells can be isolated that have low background

activity. However, steps must be taken to ensure that selection for low β-gal activity does not also select for cells that cannot be induced (see below). Alternatively, the constructs should be reintroduced into cells, taking steps to reduce copy number and expression as described above.

Poor Induction of β-gal Activity Is Observed

When induction fails to occur, flow cytometry is again useful to characterize the population. Low induction observed in a mass assay may be due to only a small part of the population responding. The FACS can be used to select for cells that have a high induced β-gal activity, but this criterion alone may result in cells that have constitutive β-gal activity. Ideally, cells will be sorted sequentially for a subpopulation or clones that have low β-gal activity in the absence of inducer, and higher β-gal activity in the presence of inducer (Blakely et al. 2000). Although a FACS simplifies this procedure, such selection is also possible simply by plating a large number of clones and then screening the clones for inducible β-gal activity.

Constitutive β-gal Activity Is Observed with Membrane Proteins

In our experience, constitutive β-gal activity is more likely to occur when the chimeric proteins are both membrane proteins. We suspect that this is because the membrane proteins, limited to the two-dimensional space of the membrane, have a higher effective concentration of complementation partners at a given level of expression than cytoplasmic proteins. Fortunately, membrane proteins offer an opportunity for another level of selection, because antibodies to the extracellular domain of the membrane protein of interest or to an extracellular domain tag can be used to select for chimera expression in live cells using the FACS (Blakely et al. 2000). Thus, instead of selecting only for low β-gal expression in the absence of inducer (ligand), which may also select for cells that fail to express the chimera, these cells can be simultaneously selected for low β-gal expression using fluorescein fluorescence and modest expression of the chimera using an antibody to the protein of interest and a secondary antibody that fluoresces at a different wavelength. We have also observed that high uninduced β-gal activity can be a problem when using a full-length receptor with a large cytoplasmic domain. In some cases, truncating the receptor so that the β-gal mutant is closer to the transmembrane domain increases the fold induction of β-gal activity primarily by reducing the background level of uninduced β-gal activity. In the case of some receptors, such as the EGF receptor, this has the added benefit of blocking ligand-induced internalization of the receptor (Blakely et al. 2000).

Continued Lack of β-gal Activity after Troubleshooting

In some cases, a lack of β-gal activity or constitutive β-gal activity may be difficult to correct. Some interactions will inevitably be inhibited by the attachment of the interacting proteins to the β-gal deletion mutant. In other cases, the interaction may occur, but the β-gal mutants may not be oriented in a manner that permits complementation. Increasing the distance between the interacting protein and the β-gal mutant using peptide linkers may help. For cytoplasmic proteins, moving the β-gal mutant from one end of the protein to the other may also improve complementation. Although a large majority of the interactions that we have tested work with this system, there is no definite way of predicting which ones will work well. However, given two proteins known to interact, it should be possible to generate a range of chimeric proteins in vitro and select those that display the best characteristics.

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Protein–protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments

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Communicated by Peter M. Howley, Harvard Medical School, Boston, MA, January 25, 2002 (received for review November 26, 2001)

We have defined inactive α and ω fragments of β-lactamase that can complement to form a functional enzyme in both bacteria and mammalian cells, serving as a readout for the interaction of proteins fused to the fragments. Critical to this advance was the identification of a tripeptide, Asn-Gly-Arg, which when juxtaposed at the carboxyl terminus of the α fragment increased complemented enzyme activity by up to 4 orders of magnitude. β-Lactamase is well suited to monitoring constitutive and inducible protein interactions because it is small (29 kDa), monomeric, and assayable with a fluorescent cell-permeable substrate. The negligible background, the magnitude of induced signal caused by enzymatic amplification, and detection of signal within minutes are unparalleled in mammalian protein interaction detection systems published to date.

Protein–protein interactions are involved in every cellular process ranging from gene expression and signal transduction to cell division and differentiation, yet they have been among the most difficult aspects of cell biology to study. Standard biochemical methods have yielded most of the available information about such interactions, but these assays often are limited by the available reagents such as monoclonal antibodies for immunoprecipitation or lack the appropriate cellular context.

The development of fusion protein-based assays such as the yeast two-hybrid method (1) has expanded the potential for studying protein interactions in intact cells greatly. However, this assay relies on the transcription of a reporter gene; consequently it is not applicable to studies of the kinetics of protein–protein interactions and is unable to detect the interaction of compartmentalized proteins such as receptors at the cell surface. A method based on fluorescence resonance energy transfer provided a further advance and currently is one of the most accurate methods used to monitor dynamic interactions (2). However, the incremental changes in fluorescence assayed by fluorescence resonance energy transfer are small, and the stringent steric requirements for detecting the interacting proteins can restrict the utility of this technique.

Assays based on the complementation of enzyme fragments fused to interacting proteins that regenerate enzymatic activity after dimerization are particularly well suited for monitoring inducible protein interactions (reviewed in ref. 3). These systems have important advantages including low-level expression of the test proteins, generation of signal as a direct result of the interaction, and enzymatic amplification. As a result, they are highly sensitive and physiologically relevant assays (4). Additionally, assays based on enzyme complementation can be performed in any cell type of interest or in diverse cellular compartments such as the nucleus, secretory vesicles, or plasma membrane.

The class A β-lactamases are particularly attractive candidates for an assay based on enzyme fragment complementation because of the fact that they are monomeric and of relatively small size (5). In addition, β-lactamases have been expressed successfully in prokaryotic and eukaryotic cells, making this system applicable to both classes of organisms (6). We identified a pair of β-lactamase fragments (α197 and ω198) that complemented to produce detectable activity in bacteria when fused to two helices that form a leucine zipper (7). Detectable interactions were not limited to these moieties, because interactions between larger proteins were detected also.

To increase the signal-to-noise ratio associated with the β-lactamase assay, we screened libraries of random tripeptides inserted at the break-point termini of the α197 and ω198 fragments for peptides that enhanced complementation of the enzyme. We report the identification of a tripeptide, Asn-Gly-Arg (NGR), that produced a profound enhancement of β-lactamase activity mediated by different protein pairs in bacteria when introduced at the carboxyl terminus of the α197 fragment.

We reasoned that extension of the β-lactamase system into mammalian cells would provide significant advantages over other fragment complementation systems currently used [e.g., β-galactosidase (8) and dihydrofolate reductase (9), because the fragments are small (<19 kDa)], there is no endogenous β-lactamase activity, and a highly sensitive cell-permeable fluorescent substrate has been developed recently (10). The β-lactamase fragments were tested to determine whether they could be used to monitor inducible interactions in a mammalian cell line measured either by fluorescence microscopy or flow cytometry. Here we show that the β-lactamase fragments also could detect inducible interactions in eukaryotic cells. Finally, the observed β-lactamase complementation was a direct measure of enzyme activity, not dependent on *de novo* protein synthesis, and generated detectable signal within minutes of protein dimerization, making it applicable to the detection of transient protein interactions. This system should have broad utility in monitoring protein interactions in diverse intracellular compartments in a range of cell types.

Materials and Methods

Construction and Screening of the Tripeptide Libraries in *Escherichia coli*. The β-lactamase α197 fragment was amplified first from pUC19 by PCR with forward primer JH101 (5′-CTGTGCCATGGTGAGTATTCAACATTTCCGTGTCG-3′) and reverse degenerate primer JH303 (5′-CTAGGAATTCMYBMYBMYBTTCGCCAGTTAATAGTTTGC-3′). The PCR product was subcloned into a p15A-based bacterial expression vector as a fusion to the carboxyl terminus of the Jun helix. TG1 cells were transformed and plated on 2xYT plates, and 1×10^4 independent colonies were collected after overnight growth at 33°C. The ω198 library was constructed similarly with forward degenerate primer JH302 (5′-GCATGGTACCVRKVRKVRKCTACTTACTCTAGCTTCCCG-3′) and reverse primer JH16 (5′-CCGGAATTCTTACCAATGCTTAATCAGTGAGGC-3′).

Abbreviations: FRB, FKBP-rapamycin binding domain of FKBP-rapamycin-associated protein; FACS, fluorescence-activated cell sorter.

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The PCR product was subcloned into a pUC-based phagemid vector between the *Kpn*I and *Eco*RI sites as a fusion protein to the amino terminus of the Fos helix. The library was transformed, and $\approx 1 \times 10^4$ independent colonies were isolated. The phagemid library was rescued with a helper phage, R408 (Stratagene), and used to infect TG1 cells expressing the α197-Jun construct including the peptide library. Infected cells (1×10^8) were plated on increasing concentrations of ampicillin (50, 100, 200, 400, and 800 μg/ml). After an overnight incubation, colonies appearing on plates containing the highest ampicillin concentrations were collected. To separate the two vectors expressing the α197 and ω198 fragments, DNA isolated from each putative clone was used to transform DH5α cells and then selected on plates containing either kanamycin or chloramphenicol for isolation of each vector expressing either the α197-peptide-Jun or the Fos-peptide-ω198 fusion protein, respectively. Colonies resistant to one antibiotic but not the other were isolated.

β-Lactamase Activity Assay in *E. coli*. Cells in 2xYT medium with 10% glucose and appropriate antibiotics were grown to a density of 0.3 OD_{600} at 33°C and then induced with 0.1 mM isopropyl β-D-thiogalactoside for 2 h without antibiotics. The culture (750 μl) was incubated with 1.5 ml of 0.1 mg/ml nitrocefin. The absorbance of 200 μl of cell-free supernatant was measured at 490 nm in a BioRad Benchmark microplate reader (Bio-Rad).

Detection of β-Lactamase Fragments in *E. coli* by Immunoblot. Cells in 2xYT medium containing 2% Glucose, 34 mg/ml chloramphenicol, and 35 mg/ml kanamycin were induced at an OD_{600} of 0.4 with 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 33°C. Cells (4×10^8) were lysed directly into SDS sample buffer or by probe sonication in PBS supplemented with 150 mM NaCl/1% Triton X-100/5% Glycerol/10 mM benzamidine/Sigma *E. coli*-specific protease inhibitor mixture/1 mM PMSF and partitioned into soluble and insoluble fractions by centrifugation. Lysates were subjected to SDS/PAGE on a 4–20% gradient gel. Proteins were Western-blotted with horseradish peroxidase-conjugated anti-Flag monoclonal antibody (Sigma) and detected by using the ECL+Plus detection kit (Amersham Pharmacia).

Retroviral Vectors for Expression of β-Lactamase Fragments in Mammalian Cells. To create the β-lactamase fusion proteins for retroviral expression in mammalian cells, an oligonucleotide encoding a GS linker, 5′-TCGAGGGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGATCGG, was inserted into the *Xho*I/*Sal*I site of both pWZL-neo and pWZL-hygro. The α197-NGR fragment was amplified by PCR from plasmid FHT 4002A1 by using the primers 5′-CTCGAGCACCCAGAAACGCTGG and 3′-GTCGACTTCCCGCCCATTTTCG. The ω198 fragment was amplified by using the primers 5′-CTCGAGGGAGTGCAGGTGGAAACC and 3′-CTCGACTTCCAGTTTTAGAAGC. The α197 fragment was cloned into the *Xho*I site of pWZL-GS-Neo, and the ω198 fragment was cloned into the *Sal*I site of pWZL-GS-Hygro. The FKBP-rapamycin binding domain (FRB) of FKBP-rapamycin-associated protein corresponding to amino acid residues 2,025–2,114 of human FRAP was cloned as an *Xho*I/*Sal*I fragment into the *Sal*I site of pWZL-α197-GS-Neo. The full-length coding sequence of FKBP12 was cloned as a *Sal*I/*Xho*I fragment into the *Xho*I site of pWZL-GS-ω198-hygro. The extracellular and transmembrane regions of epidermal growth factor receptor corresponding to amino acids 1–655 (4) was cloned as an *Nco*I/*Bam*HI fragment into the pWZL-FKBP12ω198-Hygro vector. The wild-type β-lactamase was expressed from a pWZL vector also encoding puromycin resistance, which was a generous gift from Garry Nolan (Stanford University School of Medicine, Stanford, CA).

Retroviral Production, Infection, and Mammalian Cell Culture. The ecotropic ΦNX-packaging cell line (P. L. Achacoso and G. P. Nolan, unpublished data) was transiently transfected with the proviral constructs by using fugene transfection reagent (Roche Molecular Biochemicals). The supernatant from the transfected cells was removed 48–72 h later and applied to C2C12 myoblasts. Polybrene was added to a final concentration of 8 μg/ml (Sigma). Transduced cells were selected and maintained in the appropriate antibiotic (hygromycin or neomycin, Invitrogen) at a concentration of 1 mg/ml. C2C12 myoblasts were grown in DMEM (Invitrogen)/20% FBS. Cells were treated with 50 nM rapamycin unless otherwise stated.

β-Lactamase Assayed by CCF2/AM Staining, Immunofluorescence, and Fluorescence-Activated Cell Sorter (FACS) Analysis in Mammalian Cells. *Immunofluorescence.* To assay β-lactamase in C2C12 myoblasts, the CCF2/AM substrate (Aurora Biosciences, San Diego) was used at a final concentration of 2 μM in DMEM with 2.5 mM probenecid. Cells were washed once in PBS and then incubated for 30 min with the CCF2/AM substrate at a concentration of 3×10^5 cells per ml. The plate was washed three times in PBS and visualized with a β-lactamase filter set (Chroma Technology, Brattleboro, VT; excitation 405 ± 10 nm, 425 dichroic mirror, 435-nm long-pass emission).

FACS. Data were collected on a modified FACStar plus (Becton Dickinson) with Moflo electronics (Cytomation, Fort Collins, CO). Cells were trypsinized, washed twice in PBS, incubated with CCF2/AM substrate (as described above) for 1 h, and then washed twice in a PBS/5% FBS solution. Ten thousand events were collected for each sample.

Results

Peptide Selection in *E. coli*. Chimeric proteins were constructed by using the complementing α197 and ω198 β-lactamase fragments and the leucine zipper helices from the c-Fos (Fos) and c-Jun (Jun) subunits of the AP-1 transcription factor (11). Because the Fos and Jun helices interact constitutively, they provided a system that could be used to screen libraries for peptides that enhanced interaction-dependent enzyme complementation. The leucine zipper helices were fused at the break-point termini of the β-lactamase fragments via flexible $(Gly_4Ser)_3$ linkers. The α197 β-lactamase fragment was fused to the amino terminus of the Fos helix and coexpressed with the ω198 fragment fused to the carboxyl terminus of the Jun helix (Fig. 1*A*). Synthetic oligonucleotides encoding random tripeptides were inserted into the coding sequence of each fragment between the break-point and the flexible $(Gly_4Ser)_3$ linker that connects the interacting helices to each fragment (Fig. 1*B*). The library was designed to encode only three amino acids to avoid selecting high-affinity peptides, which themselves would mediate dimerization of the fragments. The libraries also were enriched for charged amino acids, because charge–charge interactions were likely to have the greatest effect. This enrichment was achieved by using the degenerate codon VRK (cag/ag/tg) that can only encode His, Gln, Arg, Asn, Lys, Ser, Asp, Glu, and Gly.

The α197-Jun and Fos-ω198 constructs containing the peptide library insertions were cotransformed into *E. coli* cells. The cells were plated on solid medium containing a concentration of ampicillin that allowed only 0.001% of cells expressing the complementing parent fusion constructs to survive and produce colonies. Bacterial resistance to the antibiotic ampicillin is conferred through the activity of β-lactamase; therefore colony growth in the presence of ampicillin is indicative of β-lactamase activity. All colonies underwent two cycles of recovery, dilution, and replating at 10–100 cells per colony to ensure that only clones with plating efficiencies above 1% (or 1,000-fold higher than the parent) were retained. Increased resistance to ampicillin was indicative of improved β-lactamase complementation.

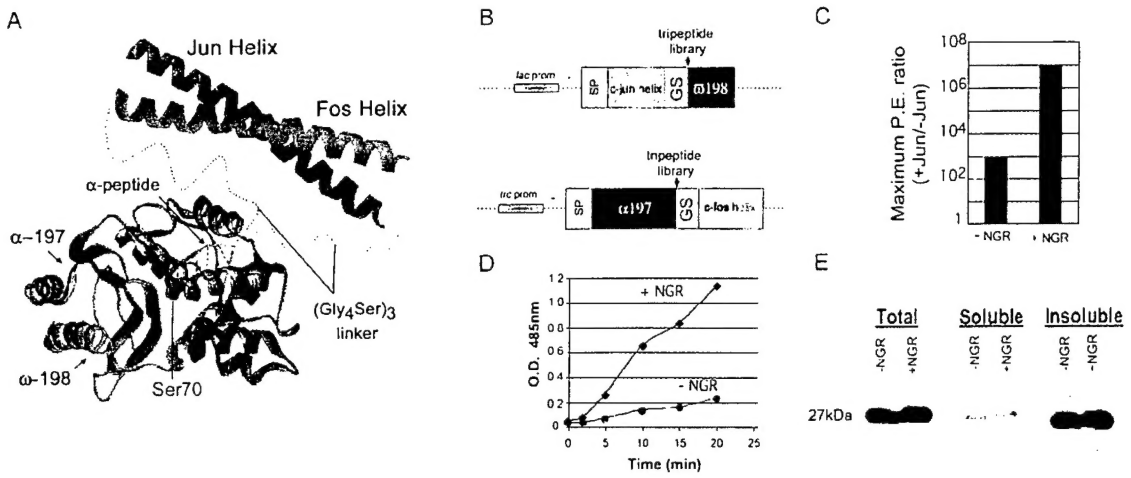

Fig. 1. β-Lactamase fragment structure and complementation assays in bacteria. (*A*) Structure of TEM-1 β-lactamase and illustrations of its reconstitution from the α197 (amino acids 25–197) and ω198 (amino acids 198–288) fragments connected by the flexible linker $(Gly_4Ser)_3$ and brought into contact by dimerization of the Fos and Jun helices. Placement of the α-tripeptide library is labeled with possible contacts as well as the active site serine (Ser-70). (*B*) Vectors used for expression of β-lactamase fragment fusion proteins with random tripeptide libraries inserted at the fragmentation point. Also indicated are signal peptide (SP), lactose operon promoter (*lac* prom), fusion of the tryptophan operon, lactose operon promoters (*trc* prom), and the glycine-serine linker (GS) as described for *A*. (*C*) Cells expressing the complementing constructs, α197Jun and ω198Fos, were plated on a range of ampicillin concentrations (10–200 μg/ml). The number of colonies obtained (signal) was divided by the number of colonies derived from plating the same constructs without the Jun helix (background) on the corresponding ampicillin concentration. The same assay was performed with the α197 fragment containing the NGR peptide insertion. The largest ratio obtained for each was designated as the maximum plating efficiency (P.E.) ratio, which is representative of five independent experiments. (*D*) Total β-lactamase activity was determined by assaying cells expressing the α197Jun and ω198Fos with and without the NGR insertion. The cells were induced to express the fusion proteins and assayed at equivalent cell densities in excess substrate (nitrocefin). Absorbance at 485 nM was determined in the cell-free supernatants of each sample and plotted against time. (*E*) To determine the effect of the NGR peptide on the stability of the β-lactamase α197 fragment, cells (from *D*) at 2 h of induction were lysed, and the accumulated protein was immunoblotted by using an antibody to the flag-tagged α197 fragment.

Twelve clones exhibiting enhanced ampicillin resistance were recovered in this manner and examined further. In all cases only the α fragment tripeptide enhanced β-lactamase activity, and no selected ω-fragment tripeptide had a significant effect either with or without its associated α-tripeptide. Four different α-tripeptides were selected: Gly-Arg-Glu (GRE), NGR, His-Ser-Glu (HSE), and Glu-Lys-Arg (EKR). Of these, the NGR peptide had the greatest effect and was chosen for further study.

Effect of Selected Peptides on Signal-to-Noise Ratio. As an indication of the selectivity of the system for interacting proteins relative to the background resulting from coexpression of the fragments, the maximum ratio of interaction-dependent activity to interaction-independent activity was determined and designated as the plating-efficiency ratio (Fig. 1*C*). Interaction-dependent activity was assessed by plating cells coexpressing the α197-Jun fusion containing the NGR peptide and the Fos-ω198 fusion on concentrations of ampicillin ranging from 10 to 200 μg/ml. The background activity, or interaction-independent activity, was measured in the same manner except that the Jun helix was removed from the α197-NGR construct. Cells expressing the parental constructs without the selected NGR peptide exhibited a 1,000-fold higher survival rate than cells in which the Jun helix had been removed, resulting in a maximum plating-efficiency ratio of 1×10^3. By contrast, the NGR peptide exhibited a maximum plating-efficiency ratio of 1×10^7. Thus, the mere presence of the NGR tripeptide between the break-point of the α197 fragment and the linker enabled a 1,000,000-fold enrichment of the Fos–Jun interaction over proteins lacking Jun (no interaction) in a single plating compared with only a 1,000-fold enrichment for the same constructs lacking NGR.

Although the increase in plating efficiency observed for the NGR peptide was encouraging, the possibility remained that the effect was specific for the Fos–Jun interaction. Previously we used the β-lactamase α197/ω198 fragment complementation system to isolate proteins that bound specifically to the extracellular domain of the human immune cell coactivation antigen CD40 [CD40ED (12)] from a library of random 12-mer peptides expressed within the context of the thioredoxin protein (ref. 13; J.-H.H. and R.B., unpublished results). The peptide showing the highest affinity for CD40ED, BW10-1, was used to test the effect of the NGR peptide on complementation mediated by this interaction.

The NGR peptide produced substantial increases in the complementation because of the interaction of BW10-1 with CD40ED over a range of ampicillin concentrations, resulting in a maximum plating-efficiency ratio that was greater than 12-fold higher than the interaction in the absence of the peptide (Table 1). Thus, although the effects of the NGR peptide on the CD40–thioredoxin protein interactions were less than on the Fos–Jun helix interaction, the enhanced ampicillin resistance

Table 1. Complementation of α197-CD40ED and BW10-1-ω198 in *E. coli*

	Ampicillin Concentration, μg/ml		
	10	25	50
+NGR, +CD40/−CD40, %	100/4.00	100/0.08	100/<0.01
Ratio	**25**	**1,250**	**>10,000**
−NGR, +CD40/−CD40, %	100/0.12	19/0.07	1.1/<0.01
Ratio	**833**	**271**	**>110**

α197 fused to BW10-1 with or without the NGR peptide was coexpressed with ω198 with or without CD40ED. Ten thousand cells expressing both constructs were plated on increasing concentrations of ampicillin, and the number of colonies formed are expressed as a percentage of cells plated. These data are representative of three independent experiments. <0.01 indicates that no colonies appeared when 10^4 cells were plated. Maximum plating-efficiency ratios are in boldface and were determined as described for Fig. 1*C*.

and signal-to-noise ratio demonstrate that the effect of the NGR peptide is not limited to the Fos–Jun interaction.

Effect of Selected Tripeptides on β-Lactamase Enzymatic Activity. To ensure that the increases in ampicillin resistance conferred by the NGR peptide were caused by increases in β-lactamase activity, we measured total enzyme activity with the chromogenic substrate nitrocefin (14). Cells expressing the complementing fusion proteins, Fos-ω198 and α197-Jun, with and without the NGR peptide were grown in suspension cultures and resuspended at the same cell density in isotonic buffer containing an excess of the substrate. The substrate and product readily diffuse into and out of the periplasm enabling the time-dependent accumulation of colored product in the cell-free supernatant (Fig. 1*D*). This assay also showed that the NGR peptide conferred a large increase in β-lactamase activity relative to controls without peptide, confirming that the observed increases in plating efficiency correlated with β-lactamase enzyme activity.

The increase in β-lactamase activity observed in the presence of the NGR tripeptide could be caused by stabilization by NGR of the α197 fragment and consequently the complemented enzyme. To determine whether addition of the NGR peptide led to accumulation of the α fragment, the amount of α197-Jun was determined by immunoblot when coexpressed with the complementing Fos-ω198 with and without NGR (Fig. 1*E*). The NGR peptide had no discernible effect on the abundance of soluble α-fragment fusion protein in either the total cell lysate or the soluble fraction, indicating that the mechanism by which NGR increases β-lactamase activity does not involve stabilizing the α197 chimera as assayed by Western blot.

β-Lactamase Complementation in Mammalian Cells. Experiments were designed to test whether the β-lactamase fragments in conjunction with the NGR peptide could be used to monitor an inducible protein interaction in mammalian cells (Fig. 2). The well characterized inducible interaction of FKBP12 and FRB was used as a model system (15–19). FKBP12 (FK506-binding protein 12) binds FRB only in the presence of the pharmacological agent rapamycin. Rapamycin is a small cell-permeable molecule that can be added directly to the culture medium resulting in heterodimerization of FKBP12 and FRB. Two fusion proteins were constructed: by using flexible linkers $(Gly_4Ser)_3$, FKBP12 was fused to the amino terminus of the ω198 fragment, and FRB fused to the carboxyl terminus of the α197 fragment containing the NGR peptide (Fig. 2*A*). The bacterial signal sequence from each of the β-lactamase fusion fragments was removed. The fusion constructs were expressed by using pWZL retroviral vectors that encode proteins conferring resistance to hygromycin or neomycin. The pWZL vectors were selected for use because they are expressed at relatively low levels; in these vectors the splice donor/acceptor is deleted, resulting in reduced translation efficiency in mammalian cells compared with other retroviral vectors such as MFG (8, 20). Thus, these vectors avoid vast overexpression of proteins and more closely approximate physiological levels.

A stable cell line containing the FKBP12ω198-hygro and α197FRB-neo constructs was established through retroviral infection of C2C12 cells and subsequent antibiotic selection. Cells from this population were treated with 50 nM rapamycin for 2 h and assayed for β-lactamase activity by using the fluorogenic CCF2/AM substrate (10). The intact CCF2/AM substrate, when excited by a UV wavelength of 409 nm, emits at 520 nm (green), whereas after cleavage by β-lactamase it emits at 447 nm (blue). As shown in Fig. 2*B* (*Top*), the cells expressing the fusion proteins appear green in the absence of rapamycin, indicating that little or no cleavage of the substrate has occurred. However, after exposure to rapamycin the substrate is cleaved, shifting the fluorescence from green to blue, indicating reconstitution of β-lactamase activity (Fig. 2*B*, *Bottom*). These results

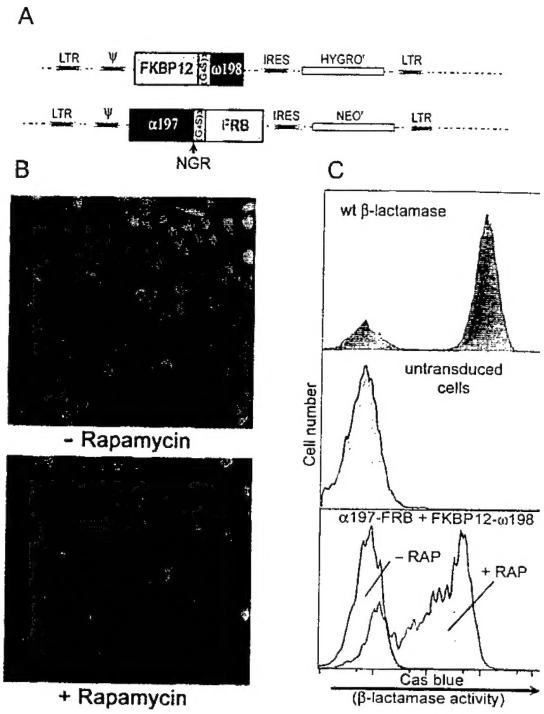

Fig. 2. Inducible β-lactamase fragment complementation in C2C1 blasts. (*A*) Schematic of the bicistronic, retrovirally expressed fusion p FKBP12ω198 and α197FRB with selectable markers for hygromycin (HY and neomycin (NEO^R) driven by an internal ribosome entry site (IR designates the viral packaging signal and LTR marks the long terminal r (*B*) Immunofluorescence assay of β-lactamase activity. C2C12 cells exp the FKBP12ω198 and α197FRB fusions were loaded with the cell-perr CCF2/AM substrate in the absence (*Upper*, −) and presence (*Lower*, rapamycin (2 h) and then imaged by fluorescence microscopy. Green in intact substrate, and blue indicates cleaved substrate. (*C*) FACS anal β-lactamase activity. Cells with and without rapamycin treatment (2 h trypsinized, loaded with CCF2/AM substrate, and assayed by flow cyto Increases in cascade blue fluorescence indicate β-lactamase activity (log (*Top*) β-Lactamase staining of cells expressing wild-type β-lactamase. (*M* Untransduced cells stained with the CCF2 substrate. (*Bottom*) Cells expr the β-lactamase fusion constructs with and without rapamycin. Dimeri of the fusion constructs induced by rapamycin causes a 50–100-fold in in cascade blue fluorescence from the responding population.

revealed that inducible dimerization of FKBP12 and FRB c lead to the complementation of the β-lactamase fragm resulting in functional β-lactamase activity in mammalian

These data were confirmed by performing a quantit measurement of β-lactamase activity by flow cytometry (F/ using the CCF2/AM substrate (Fig. 2*C*). The histograms c cells that stably expressed FKBP12ω198 and α197FRB i absence of rapamycin (*Bottom*) overlapped with and wer significantly different from untransduced negative control (*Middle*). By contrast, after exposure of the cells harborin β-lactamase fragments to rapamycin for 2 h, enzyme activity induced substantially, and an increase in fluorescence of 100-fold above background was evident. Two features of t data are particularly noteworthy: (*i*) the almost undetec background activity resulting from complementation in absence of rapamycin and (*ii*) the marked increase (orde magnitude) in the signal generated by the complementatic

Surprisingly, 20% of the cells expressing wild-typ lactamase did not stain positive for β-lactamase activity though the cells were kept in continuous drug selection to en retention of the virus containing the wild-type β-lactamase g A similar percentage of nonresponding cells (≈23%) can be

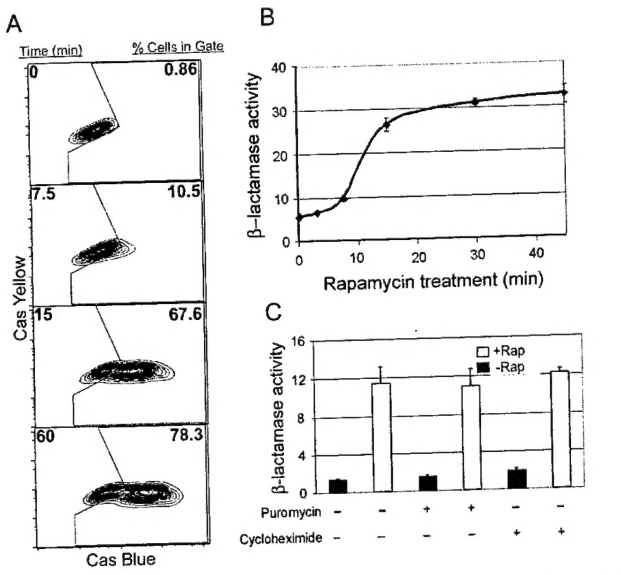

Fig. 3. Time course of inducible β-lactamase complementation in C2C12 myoblasts. (*A*) Time course of rapamycin-induced dimerization. The FKBP12ω198-α197FRB cell line was assayed for β-lactamase activity by FACS. Cells were stained with the CCF2/AM substrate, treated with rapamycin, and assayed over time. The gate is represented in the center of each plot, and the percentage of cells falling within this region are shown in red. (*B*) Mean fluorescence time course. FACS data (from *A*) represented as mean cascade (Cas) blue fluorescence were calculated in triplicate and graphed over time. (*C*) Inhibition of protein synthesis does not affect rapamycin-induced complementation. α197FRB-FKBP12ω198 cells were treated with either puromycin or cycloheximide (100 μg/ml) for 2 h before the addition of rapamycin (1 h). The cells were stained with the CCF2/AM substrate and assayed by flow cytometry. The mean fluorescence for the cascade blue channel was calculated in triplicate and graphed on the *y* axis.

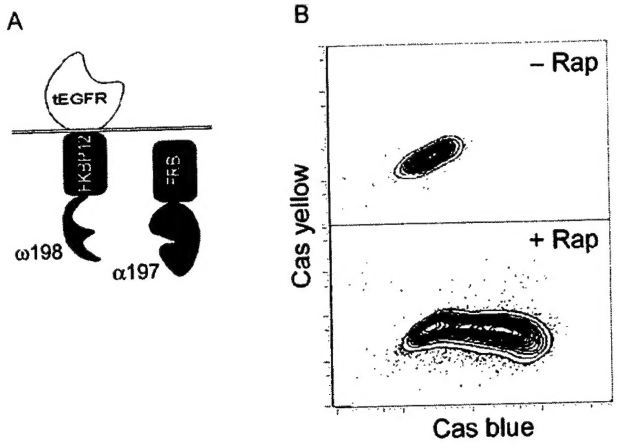

Fig. 4. Interaction of a membrane-bound and cytoplasmic protein assayed by β-lactamase complementation in C2C12 myoblasts. (*A*) Diagram depicting the membrane-bound and cytoplasmic fusion proteins that were coexpressed in C2C12 cells. The truncated epidermal growth factor receptor (tEGFR) was used to tether the FKBP12ω198 to the plasma membrane. (*B*) Cells expressing the fusion constructs in the absence (*Upper*) or presence (*Lower*) of rapamycin (1 h) were assayed by flow cytometry. Cas, cascade.

in the population of cells expressing the chimeric β-lactamase proteins in the presence of rapamycin either by flow cytometry or fluorescence imaging. This phenomenon was noted also in the original study describing the CCF2/AM substrate with similar ratios (80% responding and 20% not responding), suggesting that it may be a feature of the substrate-staining procedure itself (10). Thus 80% seems to be the maximum number of cells that can be expected to stain positive by using this assay.

Fig. 3*A* shows a time course of rapamycin treatment in C2C12 cells expressing the chimeric β-lactamase fusion proteins that demonstrated the ability to distinguish quantitatively the responding from the nonresponding population. When a gate was drawn around the cells expressing the fusion constructs before induction (time 0), it only included 0.9% of the cells. Notably, 10% of the cells were positive at 7.5 min after rapamycin addition, and at 1 h 76% of the population stained positive for β-lactamase activity, i.e., most if not all of the cells capable of responding. Longer rapamycin treatment did not increase the numbers of positive cells significantly.

The time course was rapid and began to plateau within 15 min. This result is most clearly evident when the data from a FACS analysis performed in triplicate are presented as mean fluorescence (Fig. 3*B*). After rapamycin addition, a response was seen as early as 7.5 min after rapamycin treatment and was 70% maximal within 15 min, demonstrating that by using a bulk assay for fluorescence, the generated signal is detectable also within minutes of induced complementation. These kinetics are significantly faster than those reported for other systems using the FKBP12-FRB proteins to induce dimerization (21, 22), which demonstrates the high specific activity of the complemented enzyme and the extreme sensitivity of the system.

The rapid kinetics of β-lactamase reconstitution after the addition of rapamycin suggested that *de novo* protein synthesis might not be necessary. To test this possibility, we assayed β-lactamase activity in the presence of the protein synthesis inhibitors puromycin and cycloheximide at concentrations of 100 μg/ml for 2 h before the addition of rapamycin. Neither of these inhibitors significantly altered the amount of complementation observed relative to the controls, indicating that *de novo* protein synthesis is not necessary for β-lactamase complementation (Fig. 3*C*). Many inducible protein–protein interactions have been documented to occur on a time scale of seconds to minutes. The data shown here suggest that the β-lactamase system has the potential to monitor not only rapid but possibly also transient protein–protein interactions.

Detection of Constrained Protein–Protein Interactions in Mammalian Cells. Cell surface-mediated signal transduction events often require the interaction of a membrane-associated protein such as a transmembrane receptor with a cytoplasmic protein. To test whether the β-lactamase system is capable of monitoring interactions in such a constrained configuration, we constructed a model membrane-bound protein that would interact with a cytoplasmic protein only in the presence of an inducer. For this purpose, a tripartite fusion construct comprised of the extracellular and transmembrane region of epidermal growth factor receptor, which anchored the protein to the plasma membrane fused to the FKBP12ω198 chimera (Fig. 4), was generated (4). This fusion protein was coexpressed with a cytoplasmic α197-FRB chimera as described previously. Cells expressing these constructs were assayed by FACS for induction of β-lactamase activity in the presence of rapamycin (Fig. 4*B*). The magnitude of β-lactamase complementation observed when proteins are in a constrained membrane-anchored conformation is comparable to that observed when the proteins are expressed freely in the cytoplasm (compare Fig. 3*A* with Fig. 4*B*).

Discussion

Protein fragment complementation assays have widespread potential for understanding biological processes, because they can be adapted to high-throughput assays, cDNA screens, and the study of inducible protein interactions. Such biosensors of protein–protein interactions should be invaluable in elucidating signal transduction pathways in specific cells (transformed,

differentiated, and dividing) in response to well defined extracellular stimuli such as hormones, cytokines, and calcium. Moreover, they can be used to screen for molecules that promote or disrupt such interactions, which could serve not only as invaluable biological tools but also be applied to drug discovery.

Although several systems have been developed that use chimeras of proteins of interest and enzyme fragments to assess protein interactions, each has its limitations. For example, in mammalian cells the fluorescent signal generated by complementation of dihydrofolate reductase is not amplified enzymatically; thus, only small increments in fluorescence are achieved (9, 23). The β-galactosidase system benefits from enzymatic amplification of its signal; however, the active enzyme is a homotetramer, and the individual fragments are large (80 kDa), making it likely that some interactions may be sterically hindered (4, 8). The small size, monomeric nature, and availability of a cell-permeable fluorescent substrate suggest that a system based on the β-lactamase enzyme has the potential to overcome many of the limitations of existing systems. Indeed, the β-lactamase complementation system described here exhibits an extremely high signal-to-noise ratio measured by plating efficiency in bacteria or in mammalian cells by flow cytometry and fluorescence imaging. In addition, the ability to generate a signal within minutes and the capacity to perform the assay in the absence of *de novo* protein synthesis suggests that this system may be ideal for studying inducible and transient protein–protein interactions in any cell type.

We have shown in bacteria that by screening an oligonucleotide library a tripeptide sequence could be isolated that greatly enhanced the complementation of the α197 and ω198 pair of β-lactamase fragments. The NGR peptide was shown to be effective in a leucine zipper helix interaction as well as a more complex CD40–thioredoxin protein interaction, increasing the overall sensitivity of the system by up to 3 orders of magnitude in the colony formation assay based on growth in the presence of ampicillin. Although this peptide had a direct effect on reconstituted β-lactamase activity in bacteria, the increase in activity was not caused by an increase in the stability of the α fragment or accumulation of the complemented enzyme assayed by Western blot. This finding suggests that the NGR peptide may act, at least in part, by increasing the specific activity of the reconstituted enzyme. This hypothesis is supported by analysis of three-dimensional structures for TEM-1 β-lactamase (24). We examined potential interactions between side chains of the NGR peptide and residues underlying the fragmentation site in the reconstituted enzyme. This modeling suggested that the NGR peptide could make five different contacts on three distinct helices, including two contacts on helix 2. Coincidentally, helix 2 contains the active site nucleophile Ser-70. Thus, we speculate that interactions with the helix 2 residues could stabilize the active site, increasing the specific activity of the complemented enzyme, accounting for the apparent increase in enzymatic activity.

The magnitude of complementation observed for the β-lactamase system in the presence of the NGR peptide led us to test the applicability of the system in assaying protein interactions in mammalian cells. By using the inducible FKBP12-FRB dimerization system, we showed that it is not only possible to monitor an inducible interaction in mammalian cells by using β-lactamase complementation but also that this assay yields a very robust signal of 50–100-fold increase in fluorescence from the responding cell population. This finding, as well as the negligible background observed from the expression of the fusion proteins in the absence of a dimerizing agent, makes the highly sensitive measurement of protein interactions using this system readily apparent.

Properties inherent to the β-lactamase system suggest that it approaches a physiologically relevant measure of protein interactions in mammalian cells. The α197 fragment is ≈19 kDa, whereas the ω198 fragment is only ≈10 kDa. These values are smaller than many proteins used to monitor protein localization such as green fluorescent protein, making it unlikely that the fragments will alter the function of the chimeric proteins being analyzed significantly. The assay can be performed in any cell type and can be used to assay dimerization irrespective of protein localization. In addition, we have shown that we can detect interactions in as little as 7.5 min, and that this activity can occur in the absence of *de novo* protein synthesis, demonstrating its utility in the study of inducible or transient protein interactions. In summary, we have developed a broadly applicable protein–protein interaction biosensor that has significant advantages over traditional biochemical as well as existing protein fragment complementation systems. This system should enable the identification of molecules that promote or inhibit key protein interactions via high-throughput screens in a range of cell types, phyla, and species. Moreover, given its unique properties, β-lactamase may be particularly well suited to identifying novel protein interactions specific to subcellular compartments of transformed, proliferating, and differentiating cell types via a mammalian two-hybrid assay.

We thank Julia Spiegel for invaluable technical assistance and Dr. Fabio Rossi for insightful discussions throughout this research and helpful comments on the manuscript. T.S.W. was supported sequentially by the Genentech-Stanford fellowship, National Institutes of Health (NIH) Aging Training Grant AG00259 and NIH Biotechnology Training Grant GM08142. H.M.B. gratefully acknowledges support from NIH Grants HD18179, AG09521, HL65572, and CA59717, Department of the Army Contract DAMD17-00-1-0442, and Department of Energy Subcontract 6507640.

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